UNIVERSIDADE DA BEIRA INTERIOR
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Taste receptors in the Choroid Plexus are functional and regulated by sex hormones

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Tese para obtenção do Grau de Doutor em

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(3º ciclo de estudos)

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Dedicatory

To everyone that helped me to reach this day.

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Gonçalves, I., Hubbard, P.C., Tomás, J., Quintela, T., Tavares, G., Caria, S., Barreiros, D., R.A.Santos, C.
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V. **Advances in time course extracellular production of human pre-miR-29b from Rhodovulum sulfidophilum.**
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VI. **Recombinant pre-miR-29b for Alzheimer´s disease therapeutics.**
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VII. **Identification of androgen receptor variants in testis from humans and other vertebrates.**
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I. **Experimental Tools to Study the Regulation nd Function of the Choroid Plexus**
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Chapter 13 – Blood-Brain Barrier. Neuromethods, vol 142 (pp. 205-230). Humana Press, New York
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Resumo Alargado

Os plexos coróides (PCs) são estruturas altamente vascularizadas localizadas nos ventrículos cerebrais. São constituídos por uma monocamada de células epiteliais cuboides assentes numa membrana basal que está em contacto direto com um estroma altamente vascularizado, com tecido conjuntivo rico em fibroblastos e com células do sistema imunitário. O lado apical destas estruturas está em contacto direto com o líquido cefalorraquidiano e apresenta microvilosidades e alguns cílios. O lado oposto, o lado basal, está em contacto com vasos sanguíneos altamente fenestrados. Os PCs formam vilosidades de modo a aumentar as superfícies de contacto entre as células epiteliais do lado apical com o líquido cefalorraquidiano, e com o fluído intersticial do lado basal.

Os PCs são o principal local de síntese de líquido cefalorraquidiano, estão envolvidos na vigilância imunológica do sistema nervoso central, são estruturas ativas na neurogénese e são ainda responsáveis pela remoção de compostos tóxicos e metabolitos do líquido cefalorraquidiano resultantes de processos metabólicos do sistema nervoso central. A sua ultra estrutura é composta por células unidas através de junções de oclusão que permite que este órgão forme uma barreira entre o sangue e o líquido cefalorraquidiano, a barreira sangue-líquido cefalorraquidiano, que previne o movimento de substâncias para dentro e fora do cérebro pelos espaços intercelulares. Tendo em conta a sua estrutura e localização, os PCs têm um papel crucial na monotorização da composição química do líquido cefalorraquidiano e do sangue, contribuindo para a síntese e/ou transporte de compostos essenciais para um normal funcionamento e proteção do sistema nervoso central contra agentes neurotóxicos.

A expressão de genes relacionados com a via de transdução de sinal do paladar no PC foi detetada num estudo de *microarrays* de DNA complementar, realizado previamente pelo nosso grupo de trabalho, em PCs de ratos castrados *Wistar Han*. Nesse estudo, verificou-se ainda que a via de sinalização do paladar foi uma das cinco vias mais afetadas pelas hormonas sexuais femininas, estando sobre expressa em ratos fêmea ovariectomizados. A expressão da via do paladar tem sido amplamente estudada, fora da cavidade oral, em órgãos como o estômago, intestino, pulmões, coração, testículos, artérias, entre outros. Nestes órgãos, os recetores do paladar parecem funcionar como sensores que monitorizam a composição química dos fluidos biológicos circundantes que, quando ativados, desencadeiam respostas metabólicas defensivas. A via de transdução de sinal do paladar, descrita originalmente nas células sensoriais nos botões gustativos, inicia-se com a ligação das moléculas do sabor ao respetivo recetor do paladar (Tas1r2/Tas1r3 e Tas1r1/Tas1r3, respetivamente para o doce e umami, e Tas2r para o amargo) ativando uma via de transdução de sinal que resulta na despolarização da célula. Os ensaios experimentais apresentados nesta tese tiveram como objetivo geral investigar o papel da via

do paladar na capacidade de monitorização da composição química do líquido cefalorraquidiano e/ou sangue por parte dos PCs.

Deste modo, o presente estudo teve como objetivos específicos a análise da expressão dos diferentes componentes da via de sinalização do paladar e o estudo da sua funcionalidade no PC de rato, bem como a avaliação da sua regulação pelas hormonas sexuais.

No primeiro trabalho desenvolvido, avaliou-se a expressão e a funcionalidade da via do paladar no PC de rato, por RT-PCR e *singel cell calcium imagingl*, respectivamente. Identificaram-se transcritos de genes da via de sinalização do paladar tais como os recetores do paladar *Tas1r1*, *Tas1r2*, *Tas1r3*, que formam os recetores do doce e *umami*, os recetores *Tas2r109* e *Tas2r144* que detetam compostos amargos, e moléculas da maquinaria da via de sinalização (Gustducina, fosfolipase beta 2, inositol tri-fosfato e o membro 5 da subfamília M do canal recetor de catiões com potencial transitório). A expressão das respetivas proteínas foi confirmada por *Western* blot, imunofluorescência, imunohistoquímica e imunocitoquímica. Uma análise imunocitoquímica de explantes de PC com marcação dupla da proteína alvo (Tas1r3 e Tas2r144) e do marcador de células epiteliais de PC, a transtirretina, revelou que os recetores do paladar estão localizados nas células epiteliais de PC. Uma vez confirmada a expressão de toda a maquinaria da via de transdução de sinal do paladar no PC, procedemos aos estudos funcionais. Sabendo que a maioria dos compostos tóxicos e/ou nocivos correspondem a compostos amargos, selecionámos como alvo do nosso estudo os recetores do amargo, dado que a sua presença no PC poderá estar associada à deteção de compostos neurotóxicos. De modo a avaliar a sua funcionalidade, utilizámos culturas primárias de células epiteliais de PC onde realizámos a técnica de *single cell calcium imaging* utilizando a D-Salicina como composto amargo. O estímulo com D-Salicina provocou um aumento nos níveis intracelulares do ião cálcio que na presença de um bloqueador de recetores do amargo, o Probenecid, diminuíram de intensidade. Uma vez que a presença de um bloqueador dos recetores do amargo diminui a resposta observada, é muito provável que esta se deva à ativação dos recetores do amargo. Deste modo, podemos afirmar que os recetores do amargo presentes no PC são funcionais, podendo detetar compostos amargos presentes no líquido cefalorraquidiano e/ou no sangue.

O segundo estudo desenvolvido, teve por base uma análise de dados de *microarrays* de DNA complementar de PC de ratos castrados, realizado previamente pelo nosso grupo. A análise *in silico* destes dados mostrou que o declínio das hormonas sexuais femininas aumentava significativamente os níveis de expressão dos recetores do amargo *Tas2r109*, *Tas2r124*, *Tas2r134* e *Tas2r144*, e as moléculas da via de sinalização do paladar *Plcb2* e *Trpm5*. Mostrou também que os recetores do amargo *Tas2r109* e *Tas2r144* são diferencialmente expressos entre machos e fêmeas, apresentando uma expressão mais elevada nos machos. Deste modo, no segundo estudo apresentado nesta tese, analisámos a regulação da via de transdução do paladar pelas hormonas sexuais femininas. Assim, estudámos a expressão dos genes *Tas2r109*, *Tas2r144*, *Plcb2* e *Trpm5* no PC de ratos fêmea castrados e não castrados e em explantes de PC de ratos recém-nascidos (5-6 dias de idade) incubados com diferentes concentrações de estradiol e

progesterona. Os resultados obtidos, *in vivo* e *ex vivo*, corroboram os resultados dos *microarrays* de DNA complementar comprovando a regulação hormonal dos genes da via de transdução de sinal do paladar no PC. O efeito das hormonas sexuais na resposta das células do PC a um estímulo amargo foi avaliado por *single cell calcium imaging* com o composto Benzoato de Denatónio, um ligando do recetor do amargo Tas2r144, numa linha celular imortalizada de células epiteliais de PC (Z310) em presença de estradiol e/ou progesterona e também na presença dos bloqueadores dos recetores nucleares das hormonas. Observámos que a presença de estradiol e/ou progesterona diminuiu a amplitude de resposta das células Z310. O estudo com os bloqueadores hormonais permitiu-nos concluir que o efeito do estradiol parece ocorrer via recetor nuclear, e os resultados com progesterona sugerem o envolvimento dos recetores membranares da progesterona. Com o objetivo de analisar se as respostas observadas por *single cell calcium imaging* ocorrem via recetor Tas2r144, realizaram-se ainda ensaios de cálcio com o recetor Tas2r144 silenciado com um RNAi específico. Os ensaios de cálcio após silenciamento do *Tas2r144* mostraram uma redução significativa da resposta ao Benzoato de Denatónio, de maneira semelhante aos efeitos do estradiol e progesterona, sugerindo que as hormonas sexuais femininas regulam negativamente as respostas do PC a estímulos químicos, reduzindo a Tas2r144. A regulação exercida pelas hormonas sexuais na resposta a compostos amargos no PC, eventualmente neurotóxicos, poderá estar envolvida na diferença no aparecimento e progressão de doenças do sistema nervoso central entre géneros.

Em suma, os nossos resultados confirmam a presença da maquinaria molecular da via de transdução de sinal do paladar no PC de rato, mostrando que a via do amargo está funcional, e revelam a sua regulação pelas hormonas sexuais femininas. Os resultados obtidos neste trabalho suportam a hipótese de que a via de transdução do paladar poderá ser um dos mecanismos utilizado pelo PC para monitorizar o conteúdo químico do líquido cefalorraquidiano e/ou sangue e responder de modo a modular e manter a homeostasia do sistema nervoso central. No futuro, os resultados apresentados poderão contribuir para uma melhor compreensão dos mecanismos por detrás da função do PC em monitorizar/proteger o sistema nervoso central.

Palavras chave

Plexo coroide; barreira sangue-líquido cefalorraquidiano; via de sinalização do paladar; recetores do amargo; estrogénio; progesterona

Abstract

The choroid plexuses (CPs) are highly vascularized structures constituted by a single layer of epithelial cells that project into the brain ventricles. The CPs are the main site of cerebrospinal fluid (CSF) production and constitute the Blood-CSF Barrier (BCSFB), holding high relevance in the surveillance of the CSF chemical composition. These structures contribute for the synthesis of biological compounds essential for the functioning and protection of the central nervous system (CNS) against neurotoxic insults. The expression of taste signalling pathway components in the CPs and its regulation by sex hormones was previously determined in a cDNA microarray study previously performed by our group. Moreover, the taste signalling pathway was determined as one as the top five pathways regulated by female sex hormones. The ectopic expression of sweet, umami and bitter taste signalling in extra oral organs have been extensively studied. In these organs, the taste receptors seems to behave as sensors to assess the composition of body fluids. The expression of the taste molecular machinery and its putative regulation by sex hormones in the CP raised the hypothesis that the taste signalling pathway could be one of the mechanisms involved in the monitoring of the chemical composition of blood and CSF at the BCSFB that my differ with gender.

Considering this, we aimed to evaluate the presence and the functionality of the taste signalling pathway, as well as its regulation by the female sex hormones 17β-estradiol (E2) and progesterone (P4) in rat CP.

In the first study, the presence and functionality of the taste signalling pathway was assessed. Transcripts for the taste-related genes Tas1r1, Tas1r2, Tas1r3, Tas2r109, Tas2r144, Gustducin, Plcb2, Ip3R3 and TrpM5 were found in CPs from adult *Wistar* rats. The expression of Tas1r1, Tas1r2, Tas2r144, Gustducin, Plcb2 and TrpM5 proteins was confirmed by Western blot, immunohistochemistry and immunocytochemistry. As umami and sweet receptors are heterodimeric receptors, we performed double labelling immunofluorescence, that showed the co-expression of T1R1 and T1R3 proteins that form the umami receptor, as well as, the co-expression of T1R2 and T1R3 proteins that form the sweet receptor. Having established the cellular localization of the taste machinery in CP epithelial cells (CPEC) we further evaluated the subcellular expression of taste proteins. For that, CPs were double labelled with antibodies for each of the taste-related proteins studied and a fluorescent marker of glycosylated surface-expressed proteins, revealing that taste-related proteins are located in the plasma membrane. After confirming the presence of the taste pathway molecular machinery, we proceed with functional assays. Considering that most of toxic/noxious compounds are bitter compounds that may exist in the CSF, we turned our attention to the bitter taste signalling pathway. Thus, to evaluate the functionality of the bitter pathway in primary cultures of CPEC we performed single cell calcium imaging assays using D-Salicin as the bitter stimulus. We observed an

increase in intracellular Ca^{2+} evoked by D-Salicin that was diminished in the presence of the bitter taste receptors (T2Rs) blocker Probenecid, suggesting that T2R in the CPs are capable of sensing bitter compounds in the CSF and/or blood.

An analysis *in silico* of our previous cDNA microarray data revealed that the decline of hormone levels in female rats upon ovariectomy clearly induced an up-regulation of the T2Rs *Tas2r109, Tas2r124, Tas2r134, and Tas2r144,* and the downstream effector molecules *Plcb2* and *Trpm5*. Moreover, *Tas2r109* and *Tas2r144* were differentially expressed between female and male, showing a higher expression in males. This data led us to the second study of these thesis where the regulation of the taste pathway by female sex hormones was analyzed. For that, we compared the expression of taste-related genes in the CPs of sham and ovariectomized female *Wistar* rats and in CPs explants from newborn rats incubated with different concentrations of E2 and/or P4. Our results confirmed the cDNA microarray data, corroborating the regulation of taste-related genes by E2 and P4. The bitter receptors *Tas2r109*, *Tas2r144*, and the taste-related genes *Plcb2* and *Trpm5* were down-regulated by ovarian hormones both *in vivo* and *ex vivo*. Functional implications of female sex hormones regulation was assessed, by single cell Ca^{2+} imaging, with the bitter compound, Denatonium Benzoate (DB), which is a known ligand of Tas2r144. Single cell Ca^{2+} imaging was performed in the immortalized CP epithelial cell line Z310 incubated with E2 and/or P4 in the presence of the respective hormone receptor blocker (fulvestrant or mifepristone, respectively). Intracellular Ca^{2+} variation, observed by single cell calcium imaging, was diminished in the presence of female sex hormones. However, while E2 effects were mediated via the nuclear E2 receptor, P4 effects were not abolished by the blocker of nuclear P4 receptor. Knocking-down *Tas2r144* with a specific siRNA effectively reduced the Ca^{2+} response to the bitter compound DB, in a similar manner to E2 and P4, suggesting that female sex hormones down-regulated the responses of CPEC to chemical stimuli by reducing *Tas2r144*.

In summary, our results confirmed and characterized the presence and functionality of the taste signalling machinery in CPs showing its regulation by female sex hormones. These results suggest that the taste signalling pathway may be one of the mechanisms by which the CP surveys the chemical composition of the CSF and elicit responses to modulate and maintain brain homeostasis. The achievements reached with this work will contribute to a better understanding of the mechanisms underlying the sensor/protective role of CPs in the CNS.

Keywords

Choroid Plexus; blood-CSF barrier; taste signalling pathway; bitter receptors; estrogen; progesterone; single cell calcium imaging.

Thesis Overview

This thesis is organized in 5 main chapters.

The first chapter presents the introductory section.

The second chapter includes the general and specific objectives established for the work plan of this thesis.

The third and fourth chapters present the results obtained during the development of this work, that were summarized in original research papers published in international peered review journals, and are organized as follows:

Chapter III – Tasting the cerebrospinal fluid: another function of the choroid plexus;

Chapter IV – Bitter taste signalling mediated by Tas2r144 is down-regulated by 17ß-estradiol and progesterone in the rat choroid plexus.

Finally, the fifth chapter presents the concluding remarks and the mains achievements obtained during this thesis as also some of the future perspectives.

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List of Acronyms

α-Gust – Gustducin
Aß – Amyloid beta
ABC – ATP-binding cassette carrier family
ABCB1 – ATP-binding cassette subfamily B member 1
ABCC – ATP-binding cassette subfamily C
ABCC1 – ATP-binding cassette subfamily C member 1
ABCC4 – ATP-binding cassette sub-family C member 4
ABCC5 – ATP-binding cassette sub-family C member 5
ABCG2 – ATP-binding cassette subfamily G member 2
AJ – Adherens junction
AR – Androgen receptor
AT – Annealing temperature
ATP – Adenosine triphosphate
BBB – Brain blood barrier
BCSFB – Blood cerebrospinal fluid barrier
BMAL – Brain and muscle Arnt-like protein
BP – Base pair
CLOCK – Circadian locomotor output cycles protein kaput
CNS – Central nervous system
CP – Choroid plexus
CPEC – Choroid plexus epithelial cells
Cry1 – Cryptochrome 1
Cry2 – Cryptochrome 2
CSF – Cerebrospinal fluid
Cyp450 – Cytochrome P450
DHT – 5α-dihydrotestosterone
DAG – Diacylglycerol
DB – Denatonium benzoate
DMEM – Dulbecco's modified Eagle medium
E2 – 17ß-estradiol
ER – Estrogen receptor
ERα – Nuclear estrogen receptor 1 (ER alpha)
ERß – Nuclear estrogen receptor 2 (ER beta)
ERE- Estrogen responsive elements
FBS – Fetal bovine sérum

FW – Forward

G_{α}olf – Olfactory G-protein

GDNF – Glial cell line-derived neurotrophic factor

Gnb3 – G protein subunit beta 3

Gng13 – G protein subunit gamma 13

Gnat3 – G protein subunit alpha transducin 3

Gper – G protein-coupled estrogen receptor 1

GPCR – G protein couple receptor

GPX – Glutathione peroxidase

ICC – Immunocytochemistry

ICI - Fulvestrant

IGF-1 – Insulin-like growth factor 1

IGF-2 – Insulin-like growth factor 2

IF – Immunofluorescence

IHC – Immunohistochemistry

Ip3 – Inositol 1,4,5-trisphosphate

IP3R3 – Type 3 ion channels

JAM – Junctional adhesion molecules

mER – Membrane bound ER

mPR- Membrane bound PR

mPR1 – Progesterone receptor membrane component 1

mPR2 - Progesterone receptor membrane component 2

MRPs – Multidrug-related resistance proteins

MRP1 – Multidrug resistance-associated protein 1

MRP4 – Multidrug resistance-associated protein 4

MRP5 – Multidrug resistance-associated protein 5

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-dophenyltetrazolium bromide

OAT – Organic anion efflux transporters

OATP – Organic anion transport polypeptides

OCT – Organic cation efflux transporters

OVX – Ovariectomized female rats

OOX – Orchidectyomized male rats

P4 – Progesterone

PBS – Phosphate saline buffer

PCR - Polimerase chain reaction

Per1 – Period 1

Per2 – Period 2

PIP2 – phosphatidylinositol 4,5-bisphosphate

Plcb2 – Phospholipase C-beta 2

PR – Progesterone receptor

PROP – 6-n-propylthiouracil

PTC - Phenylthiocarbamide

qPCR – Quantitative polimerase chain reaction

RT– Reverse transcribed

RT – Room temperature

RU486/RU - Mifepristone

RV – Reverse

Scnn1g – Sodium channel epithelial 1 gamma subunit

SH – Sex hormones

SLC – Solute carrier family

SLC15 – Solute Carrier family 15

SLC21 – Solute Carrier family 21

SLC22 – Solute Carrier family 22

SVZ – Subventricular zone

T1R – Taste receptor type 1

T2R – Taste receptor type 2

Tas1r1/TAS1R1 – Taste receptor type 1 member 1 (rodent/human)

Tas1r2/TAS1R2 – Taste receptor type 1 member 2 (rodent/human)

Tas1r3/TAS1R2 – Taste receptor type 1 member 3 (rodent/human)

Tas2r(number)/TAS2R(number) – Taste receptor type 2 member (number) (rodent/human)

TBS – Tis-buffer saline

TJ – Tight junction

TR – Taste receptor

Trpm5 – Transient receptor potential cation channel, subfamily M, member 5

TTR – Transthyretin

UGT – UDP glucuronosyltransferase

WGA – Wheat germ agglutinin

ZO – Zonula occludens

CHAPTER I

General Introduction

1. The Choroid Plexuses

The choroid plexuses (CPs) are highly vascularized branched structures that protrude into each ventricle of the brain, constituting the blood-cerebrospinal-fluid (CSF) barrier (BCSFB) (Figure 1). The CP filters molecules/drugs from blood, determining their bioavailability in the Central Nervous System (CNS). Furthermore, the CP produces the CSF and synthesizes and secretes a wide range of compounds, essential for brain homeostasis, making it an instrumental player in brain development, neuroprotection, neurorepair, immune and chemical surveillance of CSF composition and detoxification (Brightman and Reese, 1969; Pardridge, 2012).

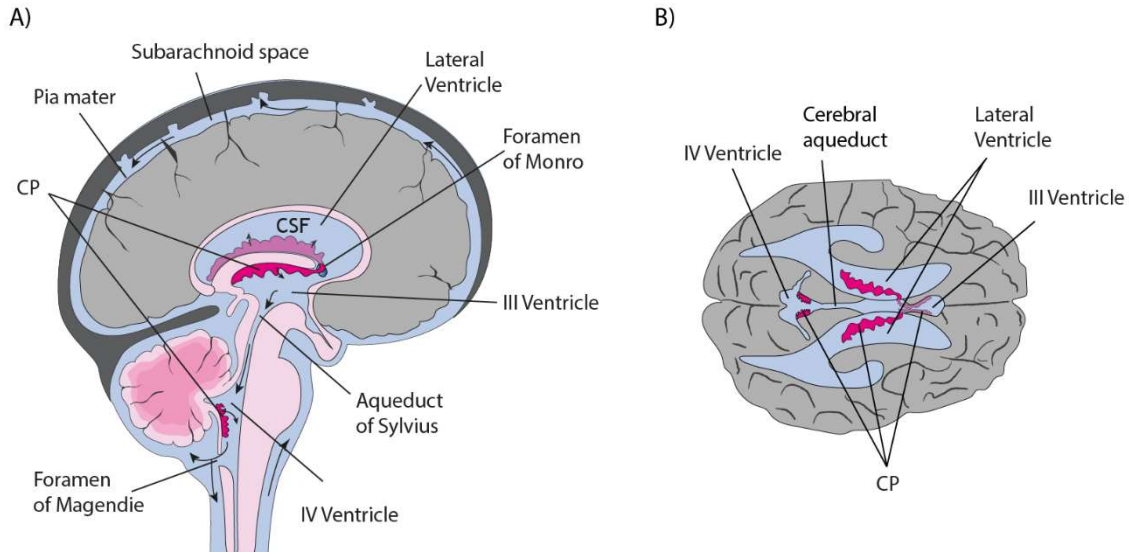


Figure 1 - Brain Choroid Plexus localization and CSF circulation. A) Sagittal section: the CSF secreted in the lateral ventricles flows through the Foramen of Monro into the third ventricle. From here the fluid flows through the aqueduct of Sylvius to the fourth ventricle. CSF then leaves the ventricular system via the foramen of Magendie into the subarachnoid space. In the subarachnoid space, the CSF is no longer in contact with the ependyma but is now separated from the outer surface of the brain by the pia mater. B) Axial section: localization of CPs in the lateral, III and IV ventricles. CP – choroid plexus; CSF - cerebrospinal fluid. (Adapted from Santos et al., 2017)

1.1. Structure of the choroid plexus

The CPs have a relatively simple structure and its ultrastructure is similar in the lateral, third and fourth ventricles. The CPs are constituted by a single layer of cuboidal to low cylindrical epithelial cells, the choroid plexus epithelial cells (CPECs), which lie on a basal membrane that is in contact with a highly vascularized stroma of connective tissue rich in fibroblasts and cells of the immune system (Figure 2).

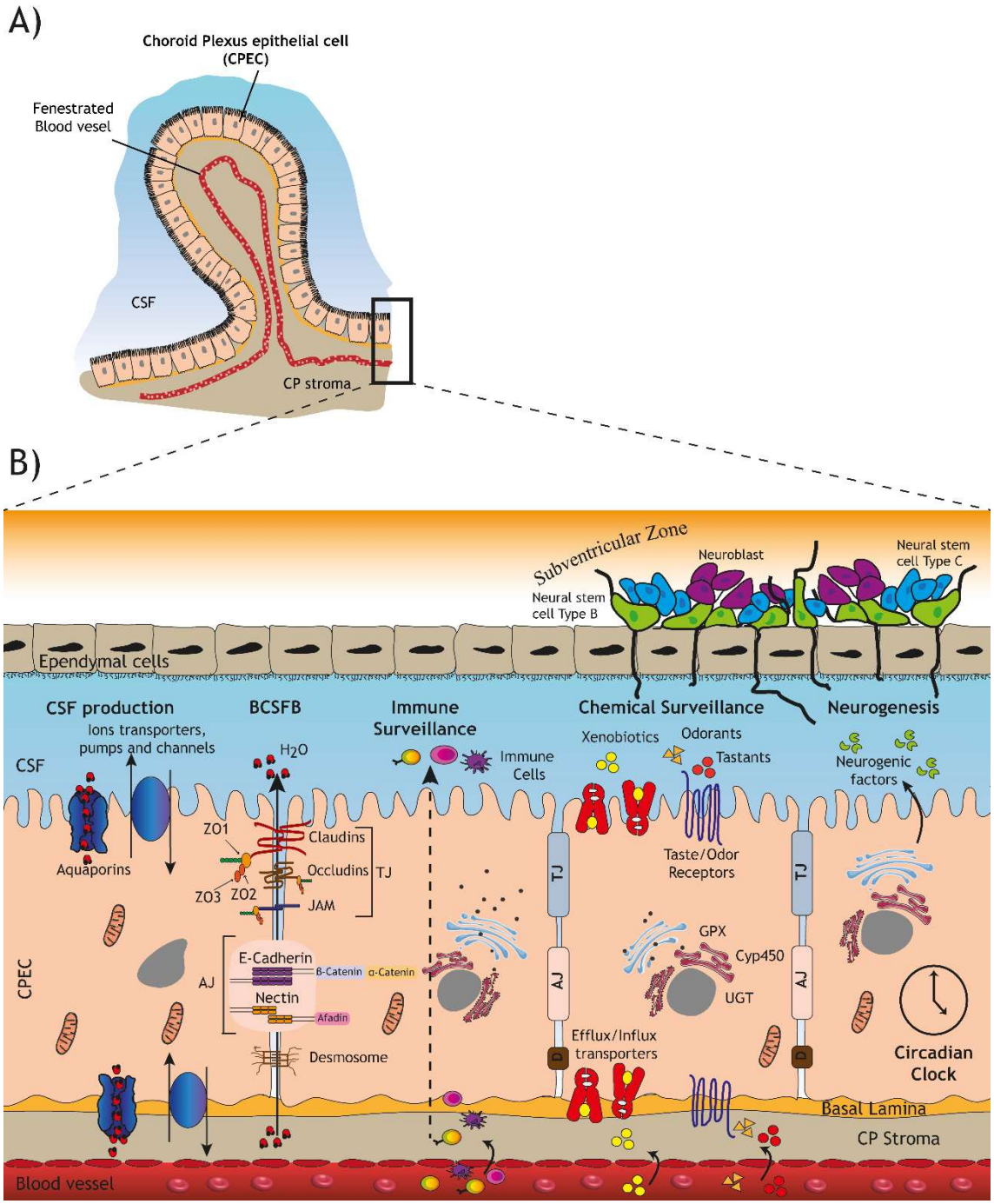


Figure 2 – Structure and main biological functions of the choroid plexus. (CP – choroid plexus; CSF – cerebrospinal fluid; CPEC – choroid plexuses epithelial cell; TJ – tight junction; AJ – adherens junction; ZO – zonula occludens protein; JAM –junctional adhesion molecules; Cyp450 – cytochrome P450; GPX – glutathione peroxidase; UGT – UDP glucuronosyltransferase). (Adapted from Santos et al., 2017)

The CPECs have a large mitochondrial content spread throughout the cytoplasm, a central nucleus, and abundant Golgi apparatus located laterally and toward the ventricular lumen consistent with the high energy demand for transepithelial transport. The surface area of the apical side (CSF facing membrane) of the CPEC is greatly enlarged by the presence of microvilli and cilia, and extensive infolding at the basolateral side (blood facing membrane). With this

structural arrangement the CP provides a large surface contact between the epithelium and the CSF, and between the epithelium and the stroma interstitial fluid. CPs are connected by tight junctions (TJ), adherens junctions (AJ) and desmosomes at the apical membrane forming a sealed barrier that prevents paracellular movement of substances in and out of the brain. Beneath the epithelial basal membrane, in stroma, there is a network of fenestrated capillaries that permit ions, water, and small molecules (e.g., nutrients, vitamins, etc.) to pass relatively easily into the interstitial fluid of the stroma of each CP (Figure 2A). It is, therefore, the monolayer of epithelial cells that constitute the BCSFB (Damkier et al., 2013; Marques et al., 2016; Mortazavi et al., 2014; Spector et al., 2015a).

1.2. Functions of the choroid plexus

Several fundamental functions have been attributed to the CPs and have been within the scope of recent reviews. The best known functions of CPs are CSF formation (Damkier et al., 2013), nutrient and hormone supply to the CSF and brain, neurogenesis (Falcao et al., 2012; Johansson, 2014; Lun et al., 2015), immune surveillance (Schwartz and Baruch, 2014) and clearance of deleterious compounds such as amyloid beta and waste products from brain metabolism (Johanson et al., 2011; Pahnke et al., 2014; Pascale et al., 2011; Richardson et al., 2015; Spector et al., 2015a). Other emerging functions of the CPs are the potential function of the CP as an extra-suprachiasmatic nucleus circadian clock (Quintela et al., 2015) and the chemical surveillance as depicted from the presence of the taste and olfactory transduction pathways in CPEC (Gonçalves et al., 2016; Tomás et al., 2016)(Figure 2B).

1.2.1. Cerebrospinal fluid production

The CSF is mainly produced by the CPs in the brain ventricles. The CSF protects the CNS in different ways by the: metabolic homeostasis, supply of nutrients, lymphatic drainage, regulation of intracranial pressure, waste removal and as a supportive environment. The total volume of CSF in the entire human CNS is about 90-150 mL, and its turnover is fairly constant. The constant secretion of CSF contributes to complete CSF renewal four to five times per day. Reduction of CSF turnover may contribute to the accumulation of metabolites seen in aging and neurodegenerative diseases (Damkier et al., 2013; Pardridge, 2016; Telano and Baker, 2018; Tumani et al., 2017). Among the CSF formed, 80% is produced by the CP in the brain ventricles and absorbed mainly via the arachnoid granulations and arachnoid villi, with the remaining 20% coming from the interstitial fluid of the brain, which is generated by the blood-brain barrier (BBB). As more CSF is produced, there is a constant directional flow from the lateral ventricles towards the fourth ventricle and beyond. Thus, the CSF secreted in the lateral ventricles flows through the Foramen of Monro into the third ventricle. From here the fluid flows through the aqueduct of Sylvius to the fourth ventricle. The CSF then leaves the ventricular system via the foramen of Magendie into the subarachnoid space where it will be reabsorbed (Figure 1A)

(Damkier et al., 2013) and will make exchanges with the interstitial fluid through periarterial influx at the Virchow-Robin spaces. This system provides a drainage pathway for the clearance of waste molecules from the brain and a site for the interaction of the systemic immune system with the brain (Brinker et al., 2014; Spector et al., 2015b).

The mechanism of CSF formation relies on the active production of an osmotic gradient. Facing the blood and the CSF, CP enables the transport of osmotically-active ions that precedes the movement of water from plasma to the CSF. Nowadays, it is consensual that the osmotic gradient in CP is driven mainly by water channels, like aquaporin 1 and ion transporters (K^+/Cl^- cotransporter and Na^+/K^+-ATPase) at the apical membrane (Brinker et al., 2014; Damkier et al., 2013). While at the basal interstitial side, Na^+ and Cl^- are carried into CPEC by membrane-bound ion exchangers (Cl^-/HCO^{3-} and the Na^+ linked Cl^+/HCO^{3-} importer)(Damkier et al., 2010; Spector et al., 2015b).

The CSF is constituted by inorganic ions (Na^+, Cl^-, HCO^{3-}, Ca^{2+}, Mg^{2+}, Mn^{2+} and PO^{4-}) and is a rich source of proteins, lipids, hormones, cholesterol, glucose, microRNAs, and many other molecules and metabolites that influence a multitude of CNS functions (Kennedy et al., 2017; Lehtinen et al., 2013). Some of the CSF constituents are actively transported into the CSF from the blood, such as albumin, immunoglobulins, folate, vitamin C, prolactin, leptin, insulin-like growth factor 1 and ascorbic acid or synthesized in the CP as transthyretin (TTR), insulin-like growth factor 2, brain-derived neurotrophic factor and then transferred into the CSF (Spector et al., 2015a, 2015b).

The composition of the CSF shows a high dynamic range, with blood and brain-born molecules, in both sides of the barrier, being able to alter the CP secretome and modulate CSF composition accordingly (Marques and Sousa, 2015; Tumani et al., 2017).

1.2.2. Barrier properties of the choroid plexus

The CP constitutes the BCSFB and, therefore, it serves as a checkpoint to limit the passage of substrates from the blood to the CSF, inhibiting paracellular diffusion, thereby protecting the CNS. Despite the fact of CP being highly permissive to a large range of molecules, the exchanges across the choroidal epithelium are highly regulated by the combined action of TJs, transport processes and metabolic enzymes. The complex system that connects the CPECs prevent the entry or the expelling of numerous molecules including toxins, drugs, and other xenobiotics (Ek et al., 2012; Ghersi-Egea et al., 2018).

The intercellular adhesion complex ensuring a close contact between cells is form by TJ, which consist of a network of claudins, occludin and junctional adhesions molecules (JAM) that are connected to adaptor proteins zonula occludens 1, 2 and 3 (ZO1, ZO2 and ZO3, respectively), AJs (constituted by cadherins) and desmosomes (Figure 2B). This adhesion complex is found in the apical region of CPECs preventing diffusion of proteins between the membrane compartments. TJs and AJs are considered to have distinct functions. Since

claudins are the principal barrier-forming protein defining the barrier size-selectivity, TJs are responsible for the transport of solutes and ions inhibiting paracellular diffusion of water-soluble molecules across this barrier (Engelhardt and Sorokin, 2009; Redzic and Segal, 2004). On the other hand, AJs are specialized cell-cell junctions that are formed by cadherins and associated proteins into which actin filaments are inserted, which together with desmosomes are critical to the maintenance of tissue structure and morphogenesis (Aijaz et al., 2006; Redzic, 2011). Molecular studies revealed that CPECs express several TJ proteins such as caludins (i.e. claudin-1, -2, -3, -5, -6, -7, -9, -11, -12, -19, -22), occludin, JAM2, JAM3, the ZO-proteins and also the AJ proteins cadherin 1 (also named E-catherin) and 22 (Kratzer et al., 2012; Liddelow et al., 2013; Quintela et al., 2013).

Along with its anatomical function as a barrier, given by TJ, the CP is also a dynamic tissue that express multiple transporters, receptors and enzymes allowing the directed transport of ions and nutrients into the CSF and the removal of toxic agents from the CSF (Engelhardt and Sorokin, 2009; Redzic and Segal, 2004; Strazielle and Ghersi-Egea, 2015, 2013). The main choroidal drug transporters that accept drugs and xenobiotics as substrates belong to two superfamilies of transporters, the solute carrier (SLC) family and the ATP-binding cassette (ABC) carrier family (Strazielle et al., 2004). The transport across the BCSFB is regulated by plasma membrane transporters working either in the blood to CSF direction, in the CSF to blood direction, or both. The directionality of the transport is defined by the basal or apical localization of the transport system in CPECs and by the transport mechanism being primarily energy dependent (ABC transporters), secondarily energy dependent (SLC transporters coupled to to Na^+/K^+-ATPase) or via facilitated diffusion (SLC transporters depending in the concentration gradient) (Redzic, 2011).

ABC systems are efflux transporters found in BCSFB and include the ATP-binding cassette subfamily B member 1 (ABCB1, also called P-glycoprotein – Pgp, multidrug resistance protein 1 – MDR1, or CD243), ATP-binding cassette subfamily G member 2 (ABCG2, also called breast cancer resistance protein, BCRP), and several ATP-binding cassette subfamily B (ABCCs, also called multidrug-related resistance proteins, MRPs) located mainly at the blood-facing membranes. These transporters are responsible for the efflux of toxic compounds or drugs from brain and CSF into blood and for the trafficking of some endogenous compounds such as certain hormones (Bernstein et al., 2014; Roberts et al., 2008; Strazielle and Ghersi-Egea, 2015). However, some ABC transporters, such as MRP1, MRP4 and MRP5 are predominantly located at the apical membrane of the CP epithelium, which localization indicates the transfer of deleterious compounds out of the epithelial cells into the CSF (Roberts et al., 2008). This suggests that the direction of efflux is from the inside of the epithelial cells into the stroma of CP or CSF, thus contributing to blood–CSF barrier function (Ek et al., 2010).

The SLC superfamily are multispecific transporters responsible for the neuroprotective efflux of exogenous and endogenous toxic molecules from brain or CSF to blood. These transporters belong mainly to three subfamilies: the SLC22 subfamily which includes organic anion (OATs)

and cation (OCTs) efflux transporters; the SLC21 subfamily that comprises another subfamily of organic anion transport polypeptides (OATPs); and the SLC15 subfamily, known as peptide/proton cotransporters. OATs and OCTs transporters accept a broader range of substrates. OATs mediate the transport of large, amphipathic solutes such as bile salts, thyroid hormones, leukotrienes, and various steroid conjugates and xenobiotics, while substrates for OCTs include neurotransmitters (serotonin, dopamine, choline, tetraethylammonium ion, cimetidine and N1-methylnicotinamide). OATPs transport endogenous di- and tripeptides, some peptidomimetic drugs, e.g., ß-lactam antibiotics, antiviral nucleoside prodrugs or angiotensin converting enzymes (Grube et al., 2018; Redzic, 2011; Saunders et al., 2016; Strazielle and Ghersi-Egea, 2015).

Transport mediated by SLC transporters can be bidirectional, or unidirectional. The SLC subfamily SLC21 (OATPs) transport both molecules from the CSF or blood into CPEC, as well as from the CPEC cytoplasm to CSF or blood, according to apical or basal localization of the transporter, respectively. Regarding, OATs and OCTs (SLC22 subfamily), these are influx transporters. They transport molecules from the CSF and blood into the CP epithelium, where they will be further transported to the blood stream or CSF, respectively, or metabolized to a less toxic compound by detoxification enzymes such as cytochrome P450 (Cyp450) (Redzic, 2011; Roberts et al., 2008).

The presence of ABC and SLC transporters at the BCSF membrane difficult the entrance of a large number of solutes and xenobiotics into the CNS. The deregulation of the transport mechanisms across the BCSFB is involved in several CNS pathologies, such as epilepsy, ischemia, Alzheimer's disease, Parkinson's disease, inflammation, depression, brain tumour, mental retardation, and brain Human immunodeficiency virus among others (Engelhardt and Sorokin, 2009; Löscher and Potschka, 2005; Strazielle and Ghersi-Egea, 2013).

1.2.3. Neurogenesis

Neurogenesis in the brain is originated from precursor cell located in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) located in the walls of the lateral brain ventricles (Miranda et al., 2017). The niche of neural stem cells located in the SVZ contact with the CSF, by extending a thin cilium between ependymal cells that is likely involved in signal transduction (Lim and Alvarez-Buylla, 2016). The CSF composition is critical for fetal development and adult neurogenic maintenance requiring an outside regulation from factors secreted by the CP into the CSF (Johanson et al., 2011).

In vivo, neurogenesis is regulated by a variety of molecules, such as hormones, growth factors, neurotransmitters and also by brain injury such as cerebral ischemia or traumatic brain injury (Johanson et al., 2011; Schänzer et al., 2004). There are several signalling molecules in the CSF that are important for the regulation of cell differentiation at the SVZ. For example, leukemia inhibitory factor stimulates the proliferation of neural stem cells being its expression very low in normal condition; however, after brain injury its expression rapidly increases promoting the

recruitment of neural stem cells and progenitor cells, which is likely to be the first step towards regeneration (Lopez-Arenas et al., 2012). Another example is Vitamin A, which is converted to retinoic acid in CP, and once in the CSF acts as a cell differentiation promoter and inhibits cell proliferation by inducing cell cycle arrest (Janesick et al., 2015). Even more, signalling molecules that are part of the Wingless/frizzled pathway which are involved in the formation, maintenance and migration of adult neural stem cells regulating synaptic transmission and neurogenesis (Inestrosa and Varela-Nallar, 2015); insulin-like growth factors 1 and 2 (IGF-1 and IGF-2, respectively) and the bone morphogenic protein 5 whom stimulate proliferation of neural precursors cells; (Lehtinen et al., 2011; Silva-Vargas et al., 2016; Zappaterra and Lehtinen, 2012); and prostaglandin D2 and interleukin 1 beta whom decrease neural stem cells activation (Codega et al., 2014; Silva-Vargas et al., 2016) are secreted by the CP into the CSF.

Thus, the CP secretome contains a reservoir of signalling molecules that can promote quiescence, as well as activation and proliferation of cells in the SVZ. Interestingly, this regulation seems to be age dependent, with a decrease in neural stem cells clone formation with aging (Lehtinen et al., 2011; Silva-Vargas et al., 2016). However, if the CP provides a supportive or an instructive regulation remains unknown.

1.2.4. Immune surveillance

By sensing both blood and CSF changes, the CP serves as a neuro-immunological interface in health and pathology by integrating signals from the brain with signals coming from the circulation, thereby establishing fully functional afferent connections of the immune system with the CNS. The choroidal epithelium forms an efficient barrier, which in addition to alterations in the composition of the CSF, conveys inflammatory immune signals into the brain, contributing to the neuroprotective functions of CPs and consequently to neuroinflammation (Baruch et al., 2014; Ghersi-Egea et al., 2018; Marques et al., 2009b).

The CP responds to acute and repeated peripheral inflammation in a different manner. In repeated peripheral inflammatory stimuli, the signalling pathways with significant alterations are those related with leukocyte migration and with the complement cascade signalling (Marques et al., 2009b) instead of the innate immune response pathways found to be altered in acute peripheral inflammation (Marques et al., 2009a). Interferons are master regulators of innate immunity and have been implicated in multiple CNS disorder (Baruch et al., 2014; Mesquita et al., 2015). Altered expression of interferon-I and interferon-II is related to the cognitive loss found in Alzheimer's disease (Mesquita et al., 2015). However, the blockage of interferon-I signalling partially restore cognitive function and neurogenesis, and re-establish interferon-II dependent CP activity, lost in aging (Baruch et al., 2014; Mesquita et al., 2015). In turn, interferon-γ signalling control the expression of its receptors by the CP epithelium, and induces the epithelial expression of trafficking molecules regulating the entry into the CNS of the immune surveillance circulating leucocytes under physiological conditions, and for repair

following acute injury (Kunis et al., 2013). The CP is also responsible for the recruitment of monocyte-derived macrophages to the injured brain after ischemic stroke (Ge et al., 2017).

CP stroma is enriched with the CNS-specific CD4+ T cells both in health and in response to CNS trauma. The recruitment of stromal activated lymphocytes into the CSF is stimulated by its chemokine content. The presence of chemokines in CP may have a relevance in the migration of T cells across de BCSFB in neuroimmune surveillance. The excess of circulating immune suppressor cells or insufficient circulating effector T cells can each lead to suppression of the CP or its lack of activation, respectively, resulting in insufficient recruitment of inflammation-resolving leukocytes to the CNS (Baruch and Schwartz, 2013; Schwartz and Baruch, 2014; Strazielle et al., 2016)

A proper immune response by CP is required to a propped neuroinflammation response, if this response is deregulated, neuroinflammation can be augmented or diminished. For instance, in multiple sclerosis, which is a chronic progressive inflammatory disease of the CNS, an excessive migration of peripheral inflammatory cells occurs through the brain barriers, towards the CNS. Specifically, the CP represents a site for lymphocyte entry in the CSF and for CSF antigens presentation and it seems to be the initial site of T cell enter into the brain, before a further massive T-cell entry through the BBB (Reboldi et al., 2009).

Several studies have revealed that the CP expresses neuroinflammatory and immune-related molecules which are affected by several factors such as aging (Baruch et al., 2014; Mesquita et al., 2015), disease (Mesquita et al., 2015; Reboldi et al., 2009), hormonal background (Quintela et al., 2013; Santos et al., 2017) and trauma (Ge et al., 2017). These works lead to the hypothesis that in a healthy brain, CP is in the front line in the defence of the CNS against neuroinflammation, highlighting its immune surveillance function.

1.2.5. Circadian clock

The biological circadian rhythms are important because they influence many pathological conditions, such as stroke, asthma, and cardiovascular or metabolic dysfunctions. There is also increasing evidence that some cancers like breast, colorectal, and blood cancers (Reszka and Zienolddiny, 2018), and neurodegenerative diseases such as Alzheimer's disease (Duncan, 2019; Myung et al., 2018) may be linked to circadian disruptions.

The circadian rhythm have an endogenous periodicity of ≈ 24 h and its mechanism consists of a network of transcriptional translational feedback loops in gene expression that involves a limited number of clock genes in the expression of core clock components (Ko and Takahashi, 2006; Reppert and Weaver, 2001).

The expression of the canonical clock genes period 1 (Per1), period 2 (Per2), cryptochrome 1 (Cry1), cryptochrome 1 (Cry2), brain and muscle Arnt-like protein isoform 1 (BMAL1) and circadian locomotor output cycles protein kaput (CLOCK) as well as their cognate proteins were described in CPs (Quintela et al., 2015). In addition, Quintela and co-workers showed that the

CP harbours an intrinsic circadian rhythmicity that persists 6-7 days after organ extraction (Quintela et al., 2018). Another study observed a faster oscillation of clock gene expression in CP compared to the suprachiasmatic nuclei, regulating CSF production and contributing to timed production and clearance of the CSF (Myung et al., 2018). However, the study of CP rhythmicity is still very poor and deserves careful attention in the future.

1.2.6. Chemical surveillance

Despite being responsible for CSF production and clearance, the CPs serves as a barrier by separating the blood from the CSF (BCSFB), secretes various growth factors that maintain the stem cell pool in the SVZ and it responds to brain trauma and inflammation. In such way it is unquestionable that the CP responds to CNS needs according to the chemical composition of blood and CSF.

1.2.6.1. Clearance of xenobiotics

The CPs have the capacity to clear potentially toxic blood- and brain-born compounds due to the action of a wide variety of drug metabolizing enzymes and antioxidant systems which together with CP transporters have impact on the clearance of xenobiotics from the CNS. The enzymatic systems responsible for drug bio-transformation exhibit a broad substrate specificity, consist of multigene families of isoenzymes, and, by functionalization and/or conjugation reactions, metabolize a large variety of molecules. Metabolic processes occurring within the cells can biotransform different types of potentially toxic compounds, producing more water-soluble, less pharmacologically or toxicologically active metabolites (Ghersi-Egea et al., 1994; Strazielle et al., 2004).

Drug metabolism classically involves three phases: phase I or functionalization phase, phase II or conjugation phase and phase III or elimination phase. In Phase I enzymes, such as members of the Cyp450 family, flavin containing monooxygenases oxidases, monoamine oxidases and NADPH–Cyp450 reductases play an important role in transforming various drugs, pesticides, dietary compounds and some carcinogenic molecules in less active compounds by creating or modifying a functional group on a lipophilic compound. In phase II enzymes like UDP-glucuronosyltransferases (UGTs), sulfotransferases, and glutathione-S-transferases, produce even less toxic and more polar compounds. Finally, in phase III metabolism products are extruded by the efflux transporters (ABC and SLC family) present at the CPs. During phase I and II of drug metabolism, reactive intermediates as epoxides, electrophilic drug metabolites or oxygenated free radicals can be formed. These reactive intermediates are inactivated by epoxide hydrolase, and by antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (GPX) also present in CP (Kratzer et al., 2013; Strazielle et al., 2004).

Several cerebral enzymes known to metabolize drugs and other exogenous compounds are located predominantly in the interfaces between blood and brain and may act at these sites as an enzymatic barrier preventing the entry of various chemicals into the adjacent brain

parenchyma (Ghersi-Egea et al., 1994). The presence of phase I and II enzymes, as well as the antioxidants enzymes have been reported by several investigators in the CP (Ghersi-Egea et al., 1994; Liddelow et al., 2013; Quintela et al., 2013; Strazielle et al., 2004).

1.2.6.2. Clearance of Amyloid ß

The clearance of Aß peptide in the BCSFB is mediated by specific transport mechanisms and through further proteolysis by a family of amyloid-degrading enzymes.

The transport mechanism includes ABC transporters, the receptor for advanced glycation end products, low-density lipoprotein receptor related protein 1 and megalin/low-density lipoprotein receptor related protein 2. These transport mechanisms transport Aß alone or in association with Aß carriers such as IGF-1, apolipoprotein J, TTR and gelsolin. While the low-density lipoprotein receptor related protein 1 binds directly to Aß and promotes its clearance across the BCSFB, to the bloodstream. Megalin is involve in the uptake of Aß carriers (IGF-1, apolipoprotein J and TTR), which are synthesized and secreted into CSF by the CP, increasing Aß efflux across the choroid plexus (Kaur et al., 2016). TTR extrusion of Aß can be affected by TTR binding to metallothionein, the CPEC synthesise metallothionein 1, 2 and 3 that can interact with TTR, the TTR/ metallothionein 2 interaction interferes with TTR binding to Aß but the TTR/ metallothionein 3 interaction improves the binding, compromising or favour Aß clearance respectively (Martinho et al., 2010). Additionally, amyloid-degrading enzymes like the insulin-degrading enzyme, neprilysin, angiotensin converting enzyme and the endothelin-converting enzymes 1 and 2 regulate Aß levels by hydrolysing several peptide bonds of both Aß40 and Aß42 turning them into less neurotoxic fragments and decreasing the levels of cerebral Aß in brain (A C Duarte et al., 2016; Kaur et al., 2016; Nalivaeva and Turner, 2019). Moreover, Aß transport is also related with cholesterol homeostasis and transport at the brain barriers. Free cholesterol exchange across BCSFB favourably affects net Aß extrusion from the CNS. Dysregulation of cholesterol homeostasis/transport in CP results in dysregulation of the BCSFB and as a consequence in Aß accumulation in the CNS (Johanson, 2018).

A proper clearance of Aß is crucial to a healthy brain. The accumulation of brain Aß interferes with oxidative phosphorylation, leads to oxidative stress and morphological structural changes. This in turn induces further pathological cascades of toxicity, inflammation and neurodegeneration process. Altogether, these features trigger a cascade that leads to neuronal loss and, consequently, to brain dysfunction and the onset of neurodegenerative diseases such as Alzheimer's disease (Chong-Bin et al., 2014; A C Duarte et al., 2016; Javitt, 2013; Kaur et al., 2016).

1.2.6.3. Olfactory signalling

The presence of the olfactory signalling machinery in the CP was mentioned for the first time by Quintela et al. 2013 in a cDNA microarrays study, where they reported at least 426 genes of the olfactory signalling in the rat CP. Gonçalves et al. 2016, confirmed the expression of the olfactory signalling machinery in rat CP and confirmed the expression of 11 different olfactory

receptors (Olr) in CP the Olr540, Olr806, Olr19, Olr1266, Olr1382, Olr476, Olr522, Olr611, Olr812, Olr1082 and Olr1105 as well as the well characterized effector proteins olfactory G-protein ($G_{\alpha}olf$), adenylate cyclase 3 and cyclic nucleotide gated channel 2. The functionality of this pathway was also demonstrated in response to polyamines which are known to be present in the CSF.

The olfactory signalling machinery was first described in the nasal olfactory epithelium, where it transmits real-time sensory signals to the brain. Olfactory receptors are G protein-coupled receptors (GPCRs) that signal by elevating intracellular cyclic adenosine monophosphate (cAMP). The binding of an odorant to an olfactory receptor activates $G_{\alpha}olf$, which subsequently activates adenylate cyclase 3, leading to cAMP production. The consequent influx of cAMP activates the cyclic nucleotide gated channel 2, ultimately leading to a series of ion fluxes that produce an action potential (Dalesio et al., 2018). However, all the proteins are also expressed in multiple extra-nasal organs, as in liver, kidney, muscle or testis, being implicated in diverse biological processes including sperm chemotaxis, wound healing, hair growth, muscle regeneration, cancer cell inhibition or adiposity (Santos et al., 2019). The activation of extra-nasal olfactory receptors deploys a variety of signalling cascades, which may culminate with a protective response by the cells. For instance, the activation of olfactory receptors on skin increases keratinocyte proliferation, migration and regeneration, promoting wound healing in humans while in bladder and colon cancer its activations results in a decrease in cell proliferation and migration (Lee et al., 2019).

The growing number of traditionally non-olfactory tissues where olfactory receptors have been identified show that these receptors are involved in many other physiological roles and can also detect non-volatile molecules in body fluids. Hence, the role of the olfactory signalling machinery in the CP may be the detection of the chemical composition of blood and CSF.

1.2.6.4. Taste signalling

The first evidence of the taste signalling pathway in the CP was described by Ren et al. 2009 in mouse, identifying the taste receptors type 1 member 2 and member 3 (Tas1r2 and Tas1r3) protein in the CPEC. Later, in a cDNA microarray study, the expression of several taste-related genes in rat CP was described (Table 1) (Quintela et al., 2013).

The mammalian taste system, in mouth, detects essential nutrients, as well as toxic substances, resulting in the perception of the five basic taste varieties: sweet, umami, salty, sour and bitter (Chandrashekar et al., 2006; Chaudhari and Roper, 2010; Iwatsuki and Uneyama, 2012; Lindemann, 2001). The activation of taste signalling occurs in response to both ionic and complex compounds, and different molecular mechanisms are responsible for their detection. Salts and acids utilize apically located ion channels for transduction directly leading to depolarization of the taste cell. On the other hand, sweet, umami and bitter receptors are mediated by GPCRs, and their activation results in second-messenger signalling.

Table 1 - Homology of taste receptor in humans and rodents (taken from the NCBI database http://www.ncbi.nlm.nih.gov/homologene.(Adapted from Foster et al., 2014).

Human Gene symbol	Human Vs Rat Identity (%)		Rat Gene symbol	Rat Vs Mouse Identity (%)		Mouse Gene symbol
	Protein	DNA		Protein	DNA	
TAS1R1	74.2	79.1	Tas1r1*	90.2	91.3	Tas1r1
TAS1R2	71.2	78.4	Tas1r2	91.2	91.0	Tas1r2
TAS1R3	74.2	75.6	Tas1r3*	92.9	93.5	Tas1r3
TAS2R1	51.5	66.4	Tas2r119*	85.3	89.7	Tas2r119
TAS2R3	65.0	77.6	Tas2r137*	89.1	92.4	Tas2r137
TAS2R4	64.6	75.0	Tas2r108*	88.2	91.1	Tas2r108
TAS2R5						
TAS2R7	69.2	78.5	Tas2r130*	93.3	92.3	Tas2r130
TAS2R8						
TAS2R9						
TAS2R10	55.0	71.8	Tas2r114*	84.5	91.2	Tas2r114
TAS2R13	56.4	69.5	Tas2r121*	82.6	88.0	Tas2r121
TAS2R14	49.8	67.6	Tas2r140*	78.8	86.9	Tas2r140
TAS2R16	52.8	69.3	Tas2r118*	92.0	93.0	Tas2r118
TAS2R19						
TAS2R20						
TAS2R30						
TAS2R31	49.5	66.2	Tas2r136*	74.6	84.3	Tas2r136
TAS2R38	65.6	76.1	Tas2r138*	87.9	91.3	Tas2r138
TAS2R39	55.0	69.3	Tas2r139*	85.0	89.6	Tas2r139
TAS2R40	65.1	77.7	Tas2r144*	88.1	91.5	Tas2r144
TAS2R41	71.9	76.5	Tas2r126*	90.6	92.5	Tas2r126
TAS2R42	50.7	65.9	Tas2r145	80.0	87.6	Tas2r131
TAS2R43						
TAS2R45						
TAS2R46	50.2	65.5	Tas2r120*	82.7	88.8	Tas2r120
TAS2R50						
TAS2R60	58.9	71.3	Tas2r135*	93.1	94.1	Tas2r135
			Tas2r13*	77.6	88.3	Tas2r102
			Tas2r102*	74.9	87.1	Tas2r102
			Tas2r103*	76.6	86.4	Tas2r103
			Tas2r104*	86.1	91.9	Tas2r104
			Tas2r105*	83.9	88.7	Tas2r105
			Tas2r106*	84.4	91.0	Tas2r106
			Tas2r107*	85.3	89.6	Tas2r107
			Tas2r109*	75.0	85.2	Tas2r109
			Tas2r110*	77.2	85.6	Tas2r110
			Tas2r113*	79.0	88.3	Tas2r113
			Tas2r116*	73.4	85.0	Tas2r116
			Tas2r117	76.5	87.5	Tas2r117
						Tas2r122
			Tas2r123*	80.4	87.0	Tas2r123
			Tas2r124*	81.6	88.3	Tas2r124
			Tas2r125*	76.1	85.6	Tas2r125
			Tas2r129*	78.1	87.0	Tas2r129
			Tas2r134*	82.5	89.3	Tas2r134
			Tas2r143*	87.4	91.7	Tas2r143

*Taste receptors expressed in the Choroid Plexus detected by cDNA microarray (Quintela et al., 2013)

There are two taste GPCRs, titled taste receptors (TR), the taste receptors type 1 (T1R) and the taste receptors type 2 (T2R). The T1R class form two dimeric receptors; the taste receptor type 1 member 1 and taste receptor type 1 member 3 (T1R1/T1R3) that respond to umami compounds and the taste receptor type 1 member 2 and taste receptor type 1 member 3 (T1R2/T1R3) that respond to sweet. The T2R class respond to bitter compounds (Chandrashekar

et al., 2006). Whatever the compound which binds the TR (either umami, sweet or bitter) the signalling cascade is similar, resulting in a conformational change of the receptor and in the activation of a series of signal transducers such as the taste-specific heterotrimeric G-protein gustducin (α-Gust) formed by: G protein subunit alpha trasnducin 3 (GNAT3 in humans, Gnat3 in rodents), G protein subunit beta 3 (GNB3 in humans, Gnb3 in rodents) and G protein subunit gamma 13 (GNG13 in humans, Gng13 in rodents) subunits; which activates phospholipase C-beta 2 (PLCB2 in humans, Plcb2 in rodents) to produce inositol 1,4,5-trisphosphate (IP3). The IP3 opens the inositol 1,4,5-trisphosphate receptor type 3 ion channels (IP3R3 in humans, Ip3r3 in rodents), triggering an increase in intracellular Ca^{2+} levels which will activate the transient receptor potential cation channel, subfamily M, member 5 (TRPM5 in humans, Trpm5 in rodents), that eventually depolarizes the cell (Figure 3) (Chandrashekar et al., 2006; Chaudhari and Roper, 2010; Roper, 2014, 2013).

The discovery that TR and its downstream effectors are expressed in the CP together with several TR functions described in extra oral organs, such as airways, gastrointestinal tract and skin, which also form body barriers, suggests that the presence of the taste pathway in the CP may be crucial for its function in monitoring the chemical composition of CSF and blood.

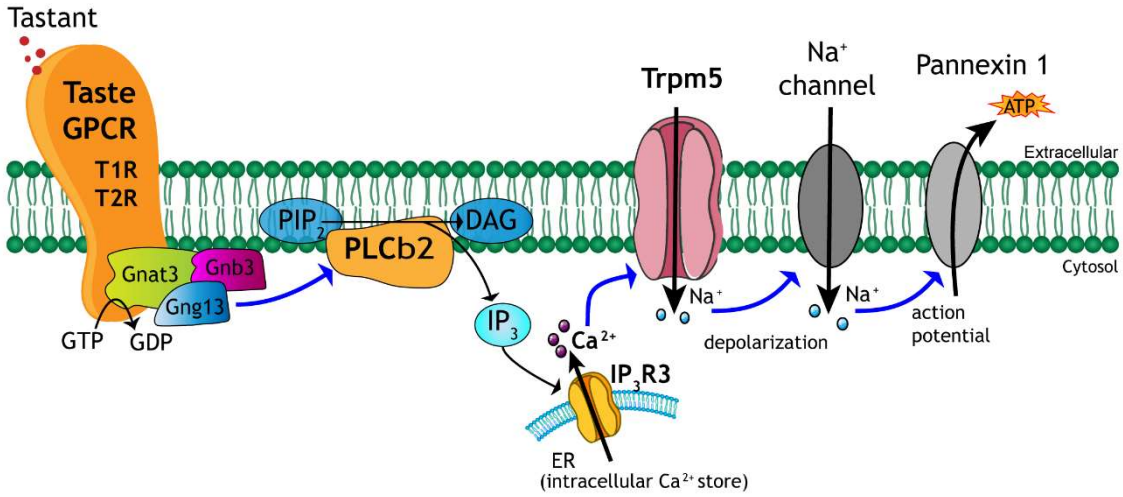


Figure 3 - Taste receptors (TR) signalling cascade. Sweet and umami compounds activate taste receptors type 1 (T1R) while bitter compounds activate taste receptors type 2 (T2R), which belong to the superfamily of G protein coupled receptors (GPCR). The taste GPCR activation results in a conformational change which results in the activation of the taste-specific heterotrimeric G-protein gustducin (formed by GNAT3, GNB3 and GNG13 subunits), that activates phospholipase C-beta 2 (PLCb2) to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) producing inositol 1,4,5-trisphosphate (IP3). The IP3 opens the inositol 1,4,5-triphosphate receptor type 3 ion channels (IP3R3), triggering an increase in intracellular Ca^{2+} levels which will activate the transient receptor potential cation channel, subfamily M, member 5 (TRPM5), that eventually depolarizes the cell. (GTP – guanosine triphosphate; GDP – guanosine diphosphate; ATP – adenosine triphosphate; GNAT3 – G protein subunit alpha trasnducin 3; GNB3 – G protein subunit beta 3; GNG13 – G protein subunit gamma 13: ER – endoplasmatic reticulum). (Adapted from Santos et al., 2019)

1.3. Taste receptors in the choroid plexus

Depending on the species, TR genes have different nomenclatures, and not all the rodent TR have corresponding human orthologs. Homology of rodents' TR with human orthologous is presented in Table 1. Henceforward, general nomenclature, T1R (taste receptor type 1) and T2R (taste receptor type 2) will be used to refer to taste receptors, and the appropriate species nomenclature: TAS1R and TAS2R for human and Tas1r and Tas2r for rodents will be used when species is referred.

Following a thorough database analysis of a cDNA microarray database several taste-related genes were found expressed in rat CP and are presented in Table 1 (GSE87045). The analysis identified transcripts of several TR as Tas1r1, Tas1r3, 32 of the 34 rat bitter taste receptors and the effector proteins Gnat3, Gnb3, Plcb2, Ip3r3, Trpm5 and sodium channel epithelial 1 gamma subunit (Scnn1g) which are involve in taste signalling cascade.

1.4. Ectopic expression of bitter taste receptors

The molecular identification of TR and their second-messenger signalling effectors in many other organs throughout the body, made it clear that taste signalling is not limited to taste buds in the oral cavity (Avau and Depoortere, 2016; Behrens and Meyerhof, 2019; Lee et al., 2019). In extra-oral organs, TRs perform additional functions including the orchestration of innate immune responses, defensive mechanisms, nutrient sensing, bronchodilatation, inflammation, metabolism, enteroendocrine regulation and affect male fertility (reviewed in Behrens and Meyerhof, 2019; Dalesio et al., 2018; Lee et al., 2019; Santos et al., 2019). In particular, bitter taste receptor (T2Rs) research has received much attention in the past years. Several studies have demonstrated the expression of T2Rs and associated signal transduction molecules in several extra-oral tissues, such as the airways, arteries, bladder, bone marrow, brain, gastrointestinal tract, heart, skin keratinocytes, kidney, leucocytes, pancreas, placenta, testis, thymus, thyroid and uterus. Moreover, activation of these receptors produces different effects in the different organs and tissues.

In the airways, T2R activation, evoke changes in respiratory rate (Finger et al., 2003; Krasteva et al., 2011; Tizzano et al., 2010), regulates innate immunity (Hariri et al., 2017; Lee and Cohen, 2015a, 2015b; Lee et al., 2014; Tizzano et al., 2010), increase ciliary motility frequency (Shah et al., 2009) and increases the secretion of antibacterial peptides to induce bronchodilation (Camoretti-Mercado et al., 2015; Deshpande et al., 2010; Lifshitz et al., 2013; Robinett et al., 2014; Tan and Sanderson, 2014).

In the gastrointestinal tract, different types of TR 'taste' the luminal content and transmit signals that regulate nutrient transport and nutrient uptake, and also the release of gut hormones and neurotransmitters involved in the regulation of energy and glucose homeostasis, limiting both food intake, gastric motility and ion secretion (Avau and Depoortere, 2016; Chen et al., 2006; Depoortere, 2014; Jeon et al., 2008; Kaji et al., 2009; Kok et al., 2018; Rozengurt,

2006; Rozengurt et al., 2006; Wang et al., 2019; Xie et al., 2018). For example, TAS2R38 activation increases ABCB1 efflux activity suggesting that TAS2R activation in gut limits the absorption of toxic substances through modulation of gut efflux membrane transporters (Jeon et al., 2011) . In diet-induced obesity mice, Tas2r108 activation stimulates glucagon-like peptide-1 (GLP-1) secretion accompanied by a decrease of circulating levels of multiple proinflammatory cytokines and chemokines that are increased in obese-diabetic mice (Kok et al., 2018). In mice intestinal tuft cells, the parasitic helminth *Trichinella spiralis* molecules activate T2R signalling to initiating type 2 immune responses. T2R activation results in interleukin 25 (IL-25) release from the intestinal villi, which activates type 2 innate lymphoid cells to produce IL-4 and IL-13 that will promote the proliferation and differentiation of stem/progenitor cells into tuft and goblet cells, resulting in tuft and goblet- cell hyperplasia (Luo et al., 2019). Hence, T2Rs play a prominent role in the communication between the lumen, epithelium, smooth muscle cells, afferent nerve fibres and the brain to trigger adaptive responses that affect gastrointestinal function, food intake and glucose metabolism (reviewed in Depoortere, 2014). These findings raise the possibility that bitter signalling in the gastrointestinal tract play a role in protecting the organism and that agonism of intestinal T2R can be therapeutic in metabolic disease such as diabetes, obesity, or diet-induced symptoms and even cancer (Depoortere, 2014; Kok et al., 2018; Widmayer et al., 2011).

T2R are also expressed in arteries, such as the mesenteric, cerebral and omental arteries, human pulmonary arteries, and accordingly to the tissue where T2R is activated, it can mediate vasodilation (Chen et al., 2017; Lund et al., 2013; Manson et al., 2014) or vasoconstriction (Upadhyaya et al., 2014). For example the T2R1 activation results in vasoconstriction in the pulmonary circuit and in relaxation in the airways (Upadhyaya et al., 2014).

In the heart, Tas2r activation (Tas2r108, Tas2r137 and Tas2r143) have a negative inotropic effect in mouse heart perfused with Tas2r agonists (Foster et al., 2014). The bitter signalling cascade activation in a keratinocyte cell line appears to influence cell differentiation (Wolfle et al., 2015). In kidney cells Tas2r105 expression plays an important role in maintaining the structure of the glomerulus and renal tubules (Liu et al., 2015). In blood from severe asthmatics humans, TAS2R pathway is upregulated in leukocytes, in which the TAS2R agonists are able to inhibit the release of several pro-inflammatory cytokines and eicosanoid release from leukocytes, suggesting to have anti-inflammatory as well as bronchodilator properties (Orsmark-Pietras et al., 2013). Also in blood, TAS2R activation participate in polymorphonuclear neutrophil chemotactic migration (Malki et al., 2015).

In pancreatic tumour and stellate cells TAS2R38 activation results in the stimulation of key transcription factors and induced up-regulation of the multidrug resistance protein ABCB1.

Therefore, influence on tumour cell biology by TAS2R38 agonists is feasible, because the response of tumour cells to chemotherapeutics might be decreased due to the over expression of the efflux transporter ABCB1 (Gaida et al., 2016).

In bladder, chloroquine, an agonist of T2R, relax human and mouse detrusor smooth muscle suppressing the overactive bladder symptoms (Zhai et al., 2016). In placental tissues, TAS2R38 is expressed and functional, namely in the syncytiotrophoblast and in the amnion (Wölfle et al., 2016). More recently TAS2R14 was also described as functional in human placenta however, its functional relevance remains unclear (Taher et al., 2019). In uterus activation of the T2R pathway in myometrial cells produces profound relaxation of precontracted myometrium in mice (Zheng et al., 2017).

In testis, ablation of Tas2r5 positive cells led to smaller testis and removed the spermatid phase from most of the seminiferous tubules (Li and Zhou, 2012). Male germ cells are likely to use T2Rs to sense chemicals in the milieu that may affect sperm behaviour and fertilization (Xu et al., 2013) and in spermatozoa T2Rs may be responsible in sensing and avoiding toxins secreted by bacteria present in the female reproductive tract (Li, 2013).

The human thyroid expresses 15 of the 25 human TAS2Rs. TAS2Rs activation in thyroid influence the production of thyroid hormones by regulating thyroid stimulating hormone (TSH)-dependent changes in intracellular Ca^{2+} and iodide efflux from thyrocytes. Interesting, some TAS2R agonist such as 6-n-propylthiouracil (PROP) and methimazole, are used clinically to reduce excess thyroid hormone production in cases of hyperthyroidism (Clark et al., 2015).

In brain, Singh et al. described the presence of transcripts corresponding to Tas2r7, Tas2r108 and Tas2r38 detected in rat brainstem, cerebellum, cortex and nucleus accumbens, and functional studies revealed an increase in intracellular Ca^{2+} after activation of T2Rs with exogenous ligands (Singh et al., 2011). Tas2r1 expression was described in rat brainstem (Dehkordi et al., 2012) and Tas2r104, Tas2r116, Tas2r118 and Tas2r138 are expressed in mouse hypothalamus, brainstem, hippocampus and cortex with Tas2r116 with different levels of expression between lean and obese mice (Herrera Moro Chao et al., 2016). TAS2R16 is expressed in human neuronal tissues and its activation in human neuroblastoma cell line SH-SY5Y modulates neurite outgrowth (Wölfle et al., 2015). TAS2R4, TAS2R5, TAS2R10, TAS2R13, TAS2R14 and TAS2R50 are expressed in human brain (Ansoleaga et al., 2013; Garcia-Esparcia et al., 2013) and differentially expressed in various brain pathologies. For instance, TAS2R10 and TAS2R13 are up-regulated in Parkinson's disease brain while TAS2R5 is down-regulated (Garcia-Esparcia et al., 2013); TAS2R5, TAS2R10 and TAS2R13 are up-regulated in Alzheimer's disease; TAS2R4, TAS2R5, TAS2R10, TAS2R13 and TAS2R14 are up-regulated in Creutzfeldt–Jakob disease; and all the six TAS2R4, TAS2R5, TAS2R10, TAS2R13, TAS2R14 and TAS2R50 are up-regulated in Progressive Supranuclear Palsy (Ansoleaga et al., 2013). The presence of the T2Rs and other proteins of the bitter taste signalling in the brain and its regulation by physiological stages implies additional functions for T2Rs apart from their gustatory function.

The extra oral tissues where the taste transduction machinery is active, seem to use the taste molecular mechanism as a sensor to assess the composition of body fluids and to respond in accordance with the activation of protective mechanisms, being therefore good candidates as targets for drug treatments.

2. Sex hormones

The sex hormones are steroid hormones which regulate a wide variety of developmental and physiological processes from fetal life to adulthood, such as promoting development and regulating metabolism. Several organs, including the adrenal gland, the testicles, the ovaries and the placenta, synthesize these steroid hormones and are known as steroidogenic organs (Miller and Auchus, 2011). Baulieu and his collaborators, in the 1980s, observed that steroids, such as pregnenolone and dehydroepiandrosterone (DHEA) are present in higher concentrations in the nervous system than in plasma, thus emerging the first evidence that the brain is a steroidogenic organ (Baulieu, 1998).

2.1. Steroidogenesis

Steroid hormones are all synthesized from cholesterol and hence have closely related chemical structures based on the classic cyclopentanophenanthrene 4-ring structure. It is a complex multienzyme process by which cholesterol is converted to biologically active steroid hormones called steroidogenesis (Figure 4).

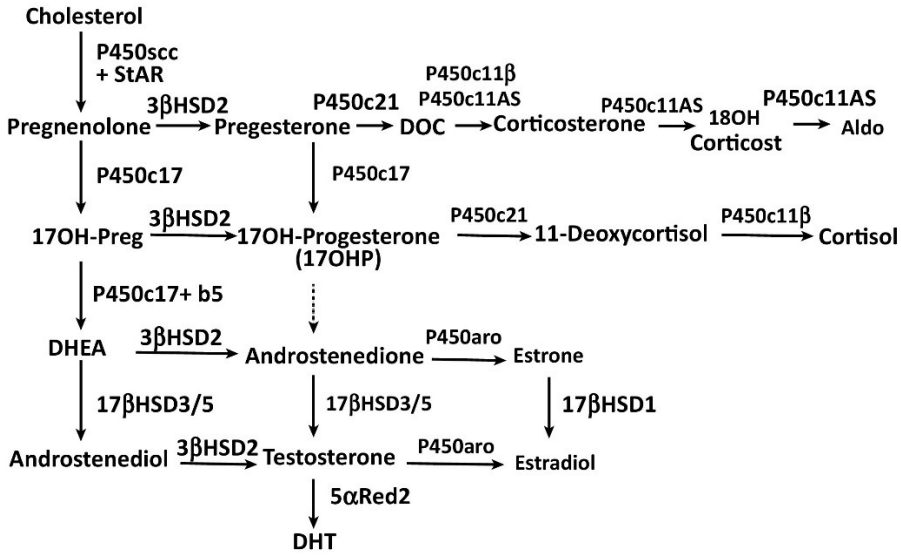


Figure 4 - Pathways of Steroidogenesis. (17OH-Preg - 17-hydroxypregnenolone; 18-OH Corticost - 18-hydroxycorticosterone; ALDO - aldosterone; DHEA - dehydroepiandrosterone; DHT - dihydrotestosterone; DOC - 11-deoxycorticosterone; P450scc - cholesterol side-chain cleavage enzyme)(Adapted from Miller, 2017)

Steroidogenesis occurs in mitochondria. Mitochondrial P450scc removes the 6-carbon side-chain of cholesterol to yield pregnenolone, a 21-carbon steroid. Hormonally inactive Δ5-steroids are converted to the corresponding Δ4-steroids by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) in the adrenal and gonad, or by the closely related 3βHSD1 in the placenta and peripheral tissues. The adrenal zona glomerulosa does not express P450c17, permitting progesterone to be 21-hydroxylated by microsomal P450c21. Mitochondrial P450c11AS (aldosterone synthase) then catalyses 11-hydroxylase, 18-hydroxylase, and 18-methyl oxidase activities to yield aldosterone (Aldo). Expression of P450c17 (17-hydroxylase) in the adrenal zona fasciculata permits synthesis of 17OH-progesterone (17OHP), which is converted to cortisol by P450c11β (11-hydroxylase). To catalyse P450c17 activity, which converts 21 carbon steroids to 19 carbon steroids, P450c17 requires allosteric action of cytochrome b5 (b5), which is essentially confined to androgenic tissues (adrenal zona reticularis and testicular Leydig cells). Even with the assistance of b5, human P450c17 converts 17OHP to androstenedione with only ~2–3% of its activity in converting 17OH-pregnenolone to DHEA, such that most human testosterone synthesis proceeds via DHEA and not via 17OHP. In the testis, 17βHSD3 readily converts DHEA to androstenediol and androstenedione to testosterone; low levels of 17βHSD5 in the adrenal zona reticularis permit the adrenal to make small amounts of testosterone. In ovarian granulosa cells (and in some peripheral tissues, especially adipocytes) P450aro (aromatase) converts androstenedione to estrone and testosterone to estradiol; in estrogenic tissues (ovary, breast, fat), 17βHSD1 converts estrone to estradiol. In genital skin (and possibly in the testis), 5α-reductase type 2 (5αRed2) further activates testosterone to dihydrotestosterone (Miller, 2017; Miller and Auchus, 2011; Miller and Bose, 2011).

2.2. Sex hormones mechanism of Action

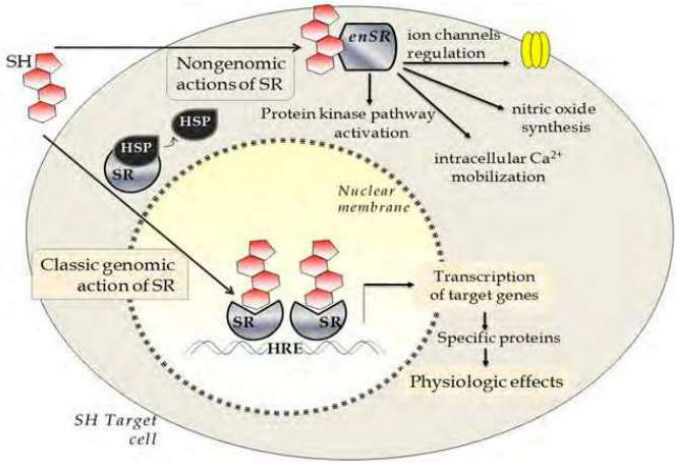


Figure 5 – Steroid hormones mechanism of Action. (SH - steroid hormones (oestrogen, progesterone); SR - steroid receptor (ER, PR) ; enSR - extranuclear steroid receptors; HSP - heat shock proteins; HRE - hormone response elements)(Adapted from Voican et al., 2012)

The mechanisms by which steroid hormones exert their effects are complex and involve both classic pathways of hormone gene transcription through their cognate receptors, as well as "non-genomic" actions (Figure 5). Genomic mechanisms involve activation of nuclear receptors in the cytoplasm which then translocate to the cell nucleus as hetero- or homodimers to bind to hormone response elements (EREs), resulting in transcription activation. Nongenomic actions occur via binding of the hormone to membrane receptors, either intracellularly or at the plasma membrane to activate second messenger systems, such as those involving mitogen-activated protein kinase (MAPK) or cyclic adenosine 3′,5′-monophosphate (cAMP) pathways, which can also activate transcription or have other effects and are characterized by significantly faster response rates (e.g. seconds, minutes)(Cole et al., 2019; Voican et al., 2012).

2.3. Sex hormones and brain disease

There is an overall acceptance that sex hormones (SH) are essential modulators of the CNS. Several brain pathologies present sex differences regarding their prevalence, symptoms and prognosis, such as Parkinson's disease (PD) (Gillies et al., 2014), Alzheimer's disease (AD) (Li and Singh, 2014), multiple sclerosis, depression and stroke (Hanamsagar and Bilbo, 2016). Both hormonal steroids released by peripheral steroidogenic glands and tissues, and steroids synthesized by neurons and glial cells (Baulieu, 1998) may contribute for these sex differences (Melcangi et al., 2009). Along with the decline of SH levels in late adulthood, neurodegeneration, is characterised by several pathological features leading to brain dysfunction and the onset of neurodegenerative diseases such as AD (A. C. Duarte et al., 2016), PD (Jurado-Coronel et al., 2018) and MS (Avila et al., 2018).

By activating cell signalling pathways that are dependent on estrogen and androgen receptors, SH are directly involved in inhibit the production and/or enhance the degradation and clearance of Aß in AD disease. The decline of SH increases the risk for development of AD (Pike, 2017). Besides women appear to have a higher incidence of AD in old age (Nebel et al., 2018), meta-analysis studies suggests that the incidence of AD is similar among men and women (Fiest et al., 2016)

Sex is also an important factor in the development of PD, being more common in men than in women by an approximate ratio of 2:1. Furthermore, the age of onset, severity and type of symptoms also differ among sexes, in PD. SH, specifically estrogens, influence PD pathogenesis and play an important role in neuroprotection in both men and women (Jurado-Coronel et al., 2018). Additionally, there are differences in the response to oral therapy (L-dopa) between gender, with men needing higher doses of L-dopa than women, to achieve optimal therapeutic control (Accolla et al., 2007).

Multiple sclerosis (MS) is a chronic inflammatory demyelination disorder with an immune-mediated pathophysiology that affects the CNS with more incidence in female gender suggesting a gender bias and a possible hormonal association. Also the female preponderance in autoimmune diseases suggests a role for hormones (Avila et al., 2018).

Notwithstanding, autism, early onset or severe schizophrenia, Tourette's syndrome, and attention deficit hyperactivity disorder (ADHD) are more common in males than in females (Reviewed by (Nelson and Lenz, 2017).

SH, in adults, exert a wide range of neuroprotective actions termed sex hormone activational effects, that decrease with age. In addition, emerging evidence suggests that developmental effects of sex steroid hormones that lead to sexual differentiation of the brain, termed organizational effects, are also involve in the onset and progression of some neuronal pathologies in adulthood (Nelson and Lenz, 2017; Pike, 2017). Clarify the mechanisms of action of sex hormones in the brain, will enhance directional therapies that recognizes sex differences in disorders and their treatment. The exclusion of sex and gender has delayed the advancement in the detection and treatment of mental/neuronal disorders. Greater attention to these differences will improve outcomes for both sexes.

3. The choroid plexus as a sexual hormone target

"(…)

The CPs are relevant multifunctional SH targets, containing SH receptors, such as progesterone receptor (PR) (Quadros et al., 2007), alpha and beta estrogen receptor (ER) (Hong-Goka and Chang, 2004) and androgen receptor (AR) (Alves et al., 2009). Their presence features the regulation of the CPs' transcriptome and secretome by sex hormones. Of notice, there are no studies on whether these may underlie many of the sex-related differences seen in neurological and neurodegenerative diseases where the CPs may be involved as recently reviewed (Marques et al., 2016).

3.1. Progesterone receptors in the choroid plexus

Progesterone (P4) improves the outcomes in animal models of traumatic brain injury, ischemia, spinal cord injury, peripheral nerve injury, demyelinating disease, neuromuscular disorders, and seizures through edema reduction, enhancement of neuronal survival, and regulation of inflammation and apoptosis (Deutsch et al., 2013). These neuroprotective and neuroregenerative effects are mediated by their cognate nuclear PR or membrane bound receptors which are expressed throughout the brain (Brinton et al., 2008). Data regarding the effects of P4 in CPs' gene expression or about PR expression in the CPs are still scarce. One of the genes, up-regulated in response to P4 is that encoding for TTR, a major thyroid hormone and vitamin-carrier protein synthesized and secreted from the CPs into the CSF (Palha, 2002), both *in vivo* and *in vitro* (Quintela et al., 2011). Nuclear PR has been found in the CPs (Table 2) and a membrane bound progesterone receptor (mPR) (Quadros et al., 2007) is present in adult CPs and other brain regions associated with osmoregulation, suggesting a role for P4 in the maintenance of water and ion homeostasis (Meffre et al., 2013, 2005).

3.2. Estrogen receptors in the choroid plexus

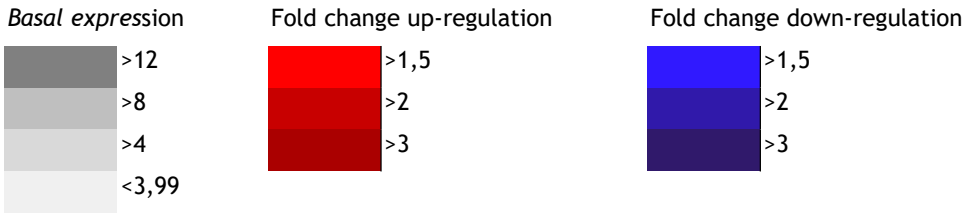
Estrogens are relevant modulators of brain functions and pathways that drive reproductive behaviour and cognition (Bridges, 2015; Engler-Chiurazzi et al., 2016). Despite the overall controversy around their benefits against aging and age-related neurodegeneration, several lines of evidence indicate that estrogens can be neuroprotective against stroke, traumatic brain injury and neurodegenerative diseases, such as Alzheimer's and Parkinson's. Estrogen actions are mediated by nuclear or membrane-bound ER, which are also expressed in the CPs. In rat CPEC primary cell cultures and in the Z310 cell line, ER beta seems to be more abundant than ER alpha, what is corroborated by data from the rat CPs' transcriptome (Table 2). Of notice,

the CPs of ovariectomized female mice display identical levels of the two ER receptors, and supplementation with E2 solely increases the expression of the ERβ (Quintela et al., 2009). Interestingly, the CPs from gonadectomized male rats showed increased expression of ERβ, suggesting that androgens may reduce ERβ in the CPs (Table 2).

Table 2 - Sex hormones receptors expressed in the choroid plexus (Santos et al., 2017).

Gene symbol	Gene name	Female sham	OVX vs Sham	Male sham	OOX vs Sham	Male vs Female
AR	*Androgen receptor*					
ERa	*Estrogen receptor 1 (ER alpha)*					
ERβ	*Estrogen receptor 2 (ER beta)*					
Gper	*G protein-coupled estrogen receptor 1*					
PR	*Progesterone receptor*					
mPR1	*Progesterone receptor membrane component 1*					
mPR2	*Progesterone receptor membrane component 2*					

OOX – orchidectyomized male rats; OVX – ovariectomized female rats



3.3. Androgen receptors in the choroid plexus

The AR is also present in the CPs of both male and female rodents. 5α-dihydrotestosterone (DHT) down-regulates AR expression in the CPs of gonadectomized male and female mice (Alves et al., 2009).

Therefore, different hormonal levels, as seen between males and females and sex hormones decline along aging are likely to set variations in CPs' gene and protein expression, including the expression of their cognate receptors, which is likely to affect CPs' functions by eliciting distinctive downstream responses in genes involved in CPs' physiology.

3.4. Functional implications of sex hormone regulation in the choroid plexus

The influence of sex hormones in the CPs' functions may be viewed from two perspectives. That strictly originating from the sex differences when comparing males and females, and that resulting from gonadectomized female and male rats, compared with the corresponding sham-operated counterparts.

Comparison of the CPs' transcriptome of male and female sexually mature rats (Quintela et al., 2016) revealed 453 up-regulated (1.8%) and 160 down-regulated (0.6%) genes in male's CPs (Table 3).

The comparison of the CPs' transcriptome of gonadectomized male and female rats to sham controls by cDNA microarrays (Quintela et al., 2013) brought forward overwhelming differences. Approximately 6000 genes corresponding to 25% of the whole rat transcriptome were differently expressed between ovariectomized female rats (OVX) and sham females among which, 1168 (4.7%) of the genes were up-regulated and 1328 (5.3%) genes were down-regulated, considering fold differences above 1.5 (Table 3). Gonadectomy did not induce so profound differences in the transcriptome of males' CPs compared to sham animals, but still 15% (549 genes) of the rat CPs' transcriptome experienced variations corresponding to 426 (1.7%) up-regulated genes, and 123 (0.5%) down-regulated genes when compared to sham animals (considering fold differences above 1.5; Table 3).

Table 3 - Number of up- and down-regulated genes in rat CP after gonadectomy and between males and females (Santos et al., 2017).

Fold-change	Females OVX vs Sham No. of genes (% of genome)	Males OOX vs Sham No. of genes (% of genome)	Males Sham vs Females Sham No. of genes (% of genome)
Up-regulated			
>1.5	1168 (4.7%)	426 (1.7%)	453 (1.8%)
Down-regulated			
>1.5	1328 (5.3%)	123 (0.5%)	160 (0.6%)

OOX – orchidectyomized male rats; OVX – ovariectomized female rats

(…)" Adapted from Santos et al., 2017

The large number of CP's genes essential to its biological functions, that are differently expressed between sexes and by the SH background, bring forward the relevance of SH for all known CPs functions (Table 4) (Santos et al., 2017).

Table 4 – Number of genes differentially expressed (>1.5 fold) by sex hormones in the choroid plexus associated with its functions. (Adapted from Santos et al., 2017)

Choroid Plexus function	Nº of genes differential expressed (>1.5 fold)		
	OVX vs Sham	OOX vs Sham	Male vs Female
Cerebrospinal fluid production			
Down-regulated	2	1	
Up-regulated	8	1	2
BCSFB			
Down-regulated	1	1	1
Up-regulated			1
Neurogenesis			
Down-regulated	15	1	17
Up-regulated	4	4	3
Immune surveillance			
Down-regulated	6		1
Up-regulated	1	1	3
Circadian rhythm			
Down-regulated	1	4	1
Up-regulated	4	1	4
Chemical Surveillance			
Clearance of xenobiotics			
Down-regulated	2	2	4
Up-regulated	2	2	3
Clearance of Amyloid ß			
Down-regulated	1		1
Up-regulated			
Olfactory signalling			
Down-regulated	5	2	
Up-regulated	5	3	3
Taste signalling			
Down-regulated			
Up-regulated	7		2

OOX – orchidectyomized male rats; OVX – ovariectomized female rats

3.4.1. Cerebrospinal fluid Production

There are several differences between males and females in CSF composition, flow dynamics and density suggesting the involvement of SH in the regulation of CSF production and osmoregulation. In fact, in a cDNA microarray study with CPs from gonadectomized *Wistar* rats, several genes associated with CSF production were differently expressed between sexes and after SH withdrawal (Table 4). Genes involved in CSF production, such as some aquaporins (aquaporin 5 and 7), ion transporters (SCL transporters), ion channels (CL^-, K^+) as well as pivotal hormone receptors and enzymes involved in CSF osmoregulation were differently expressed

according the SH background (Santos et al., 2017). Moreover, CSF protein content from male *Wistar* rats presented lower levels of fructose-bisphosphate aldolase C, prostaglandin D2 synthase, TTR, apolipoprotein A-1 and insulin growth factor binding protein 2 than CSF samples from females (Quintela et al., 2016). Interestingly, although the effect of gonadectomy in the females' CPs transcriptome was more prominent than in males' (Quintela et al., 2013; Santos et al., 2017), the CSF proteome was only influenced by gonadectomy in males (Quintela et al., 2016).

3.4.2. The Blood-Cerebrospinal Fluid Barrier

Of all transmembrane proteins present on the BCSFB, claudins, which are part of TJ, and the ones that determine the selective size, charge, and conductance properties of the paracellular pathway (Engelhardt and Sorokin, 2009; Redzic and Segal, 2004) are the only ones sensible to SH. Claudin 1 is down-regulated in OVX rats, claudin 12 is down-regulated in OOX rats an claudin 5 and 7 are differentially expressed between sexes (Santos et al., 2017). The impact of these differences in the paracellular transport across the BCSFB deserves further investigation to access differences in the effectiveness of the barrier properties between sexes.

3.4.3. Neurogenesis

Sex hormones regulate the expression of survival and proliferation genes in the CPs and therefore the activation and proliferation of cells in the SVZ. Members of the fibroblast growth factor family, cytokines, the Wingless/frizzled pathway, genes associated with cell differentiation and neuroprotection, retinoate biosynthesis and HIPPO signalling are regulated by SH in the CPs (Marques et al., 2011; Quintela et al., 2013). The reduction of SH after gonadectomy had its higher impact on females' CPs transcriptome (Table 4), but differences in the basal expression between sexes are also significant (Quintela et al., 2013).

The regulation of genes that encode pathways involved in formation, maintenance, repair and regeneration, proliferation and migration of adult neural stem cells, cell differentiation and neuroprotection by SH may be of relevance for development and/or tissue regeneration.

3.4.4. Immune surveillance

The influence of sex hormones in immune surveillance at the CPs is poorly understood. However, it is known that chemokines, adhesion molecules and cytokines transcripts are affected by gonadectomy and have different basal expression between sexes with most of them presenting higher expression in males. Also, immune surveillance related genes are more affected by gonadectomy in females than in males (Quintela et al., 2013; Santos et al., 2017). Immune surveillance and as consequence immune response by CPs is for certain a target of SH, nonetheless the functional implications of these regulation remains poorly understood.

3.4.5. Circadian clock

The CPs exhibited different circadian expression of the canonical genes of the circadian rhythm between sexes. While *Per2* and *Cry2* presented a 24-h rhythmicity in males and females, *Bmal1* and *Per1* rhythmicity was only observed in females (Quintela et al., 2015). Moreover, gonadectomy affected female and male transcriptome in the opposite way, for example, while *Bmal 1* is down-regulated in OVX and up-regulated in OOX, *Per2* and *Per3* are up-regulated in OVX and down-regulated in OOX. Interestingly the same genes are differently expressed between sham females and males (Santos et al., 2017).

The differential expression of genes responsible for setting the circadian rhythmicity between males and females, and between gonadectomized and sham animals (Table 4) suggest that sex hormones are key regulators of the CP rhythmicity. *Bmal1* mRNA circadian expression in OVX animals is ~6 h ahead than that of sham animals, and in the case of *Per1* and *Per2*, the circadian profile is completely altered, with the appearance of circadian rhythmicity in CP *Per1* values, and no circadian rhythmicity in *Per2* after OVX (Quintela et al., 2018). There is enough evidence to suggest that sex differences and/or gonadal steroids regulate the circadian oscillations of the expression of clock genes in CPs. Moreover, in neurodegenerative conditions such as Alzheimer's disease, the soluble Aß detectable in CSF shows diurnal fluctuations, with an increase during wakefulness and a decrease during sleep (Minakawa et al., 2019). Therefore, the SH circadian rhythm regulation in CPs may play an important role in neurodegenerative diseases, such as Alzheimer disease pathophysiology.

3.4.6. Chemical surveillance

When concerning the CP, three of the top five pathways differently affected by the sex hormones background are involved in chemical surveillance, the olfactory and taste signalling and the metabolism of xenobiotics by Cyp450 (Quintela et al., 2013; Santos et al., 2017).

In what concerns to chemical surveillance, while the gonadectomy effect in olfactory signalling is similarly in female and male CPs, the metabolism of xenobiotics by Cyp450 and the taste signalling are regulated by SH only in females. However, while the metabolism of xenobiotics by Cyp450 is down-regulated after OVX, the taste signalling is up-regulated (Quintela et al., 2013). Sex differences in xenobiotic detection and metabolism in CPs may lead to major differences in susceptibility to the toxicity of a xenobiotic and may affect drug safety and effectiveness (Alfonso-Loeches et al., 2013; Bourque et al., 2011).

Considering the difficulty of drugs to cross brain barriers and to target brain diseases, the discover of signalling pathways involved in chemical detection and their regulation by SH in the BCSFB is an important finding which needs further attention. Therefore, we focused our attention in the study of the taste signalling pathway in the CPs.

The taste-related genes most affected by sex-hormones in the CPs are the T2Rs. T2Rs bind bitter compounds which are mainly potentially toxic substances or therapeutic agents.

Therefore, the down-regulation of taste signalling by female SH may alter the perception of CPs to noxious compounds and consequently alter its clearance from CSF. Moreover, several drugs used in the treatment of CNS diseases are also ligands of T2Rs, as donepezil (Alzheimer's), phenytoin (Epilepsy), dexamethasone (Multiple Sclerosis), chloramphenicol, vancomycin, sulfamethoxazole (Meningitis), deferiprone (Superficial Siderosis) among others (Dagan-Wiener et al., 2019), turning T2Rs in potential therapeutically targets.

Various taste-related genes are regulated by SH in rat CPs as presented in Table 5. From this table we can highlight the Tas2Rs 107, 109, 124, 134 and 144 that are at least 1.5 downregulated by female SH and the Tas2R109 and 144 that are differentially expressed between females and males having higher expression in males.

In fact, the different clinical presentations and response to treatment of some neuronal disease between sexes, may be associated with the SH regulation of CPs transcriptome. This knowledge may also allow to develop promising targeted directional therapy.

Table 5 – Taste signalling genes differentially expressed in rat choroid plexus after gonadectomy. cDNA microarray data retrieved from NCBIs Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/, accession number GSE87045).

Gene Symbol	Gene name	Female Sham	Fold change OVX Vs Sham	Male Sham	Fold change OOX Vs Sham	Fold Change Male Vs Female
Gnb3	*Guanine nucleotide binding protein, beta polypeptide 3*	5.7	1.9	6.3		
Trpm5	*Transient receptor potential cation channel, subfamily M, member 5*	5.4		5.5	1.3	
Itpr3	*Inositol 1,4,5-triphosphate receptor, type 3*	4.4		5.1		
Plcb2	*Phospholipase C, beta 2*	3.3	1.9	3.8		
Scnn1g	*Sodium channel, nonvoltage-gated 1, gamma*	2.6	1.6	3.0		
Gnat3	*Guanine nucleotide binding protein, alpha transducing 3*	1.7	0.6	1.5		
Tas1r3	*taste receptor, type 1, member 3*	6.3		5.7		
Tas2r113	*taste receptor, type 2, member 113*	5.4		5.4	1.3	
Tas1r1	*taste receptor, type 1, member 1*	5.0	1.4	5.2		
Tas2r109	*Taste receptor, type 2, member 109*	4.6	1.5	5.2		1.5
Tas2r116	*Taste receptor, type 2, member 116*	4.6		5.1		
Tas2r135	*Taste receptor, type 2, member 135*	3.7		3.6		
Tas2r129	*Taste receptor, type 2, member 129*	3.2		3.9		
Tas2r126	*Taste receptor, type 2, member 126*	2.9		3.6		
Tas2r118	*Taste receptor, type 2, member 118*	2.7		3.5		
Tas2r136	*Taste receptor, type 2, member 136*	2.3		2.5	1.8	
Tas2r39	*Taste receptor, type 2, member 139*	2.3		2.2		
Tas2r140	*Taste receptor, type 2, member 140*	2.3		2.1		
Tas2r102	*Taste receptor, type 2, member 102*	2.0		2.3		
Tas2r121	*Taste receptor, type 2, member 121*	2.0		1.3		
Tas2r104	*Taste receptor, type 2, member 104*	2.0		2.9		
Tas2r103	*Taste receptor, type 2, member 103*	1.9	0.5	1.0		
Tas2r40	*Taste receptor, type 2, member 144*	1.9	3.4	3.3		2.7
Tas2r110	*Taste receptor, type 2, member 110*	1.8		1.5		
Tas2r108	*Taste receptor, type 2, member 108*	1.8	0.6	1.5		
Tas2r38	*Taste receptor, type 2, member 138*	1.8		2.7		
Tas2r134	*Taste receptor, type 2, member 134*	1.8	2.6	2.4		
Tas2r143	*Taste receptor, type 2, member 143*	1.7		1.5		
Tas2R130	*Taste receptor, type 2, member 130*	1.7		1.5		
Tas2r106	*Taste receptor, type 2, member 106*	1.7	1.3	1.4		
Tas2r119	*Taste receptor, type 2, member 119*	1.7		1.9		
Tas2r105	*Taste receptor, type 2, member 105*	1.7		1.5		
Tas2r123	*Taste receptor, type 2, member 123*	1.6		2.7		
Tas2r125	*Taste receptor, type 2, member 125*	1.5		2.4		
Tas2r114	*Taste receptor, type 2, member 114*	1.5		1.3		
Tas2r137	*Taste receptor, type 2, member 137*	1.4		1.9		
Tas2r13	*Taste receptor, type 2, member 13*	1.4		1.1		
Tas2r107	*Taste receptor, type 2, member 107*	1.4	1.8	1.0		
Tas2r120	*Taste receptor, type 2, member 120*	1.4	0.6	1.0		
Tas2r124	*Taste receptor, type 2, member 124*	1.4	3.0	2.7		

OVX - ovariectomized female rats; OOX - orchidectyomized male rats

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Chapter II

Global Aims

Global Aims

Despite their relevance for brain homeostasis, the mechanisms of chemical surveillance underlying the ability of the CP to monitor the composition of the CSF and blood remain unclear. The presence of the taste signalling pathway in the CP raised the hypothesis that this pathway may be one of the mechanisms involved in the monitoring of the chemical composition of blood and CSF at the BCSFB.

Furthermore, the analysis of the effects of the sex hormones background in the rat CPs transcriptome has shown that the taste signalling pathway is one of the top five pathways regulated by female sex hormones, thus suggesting that if this pathway is functional at the CP, it may be modulated by female sex hormones, E2 and P.

Thus, the main goals of this project were to analyse the expression and function of the taste-like chemosensory pathway in CP, and its regulation by sex hormones. In this framework, the following specific objectives were established:

i) To analyse the expression of the taste transduction machinery in CP: mRNA and protein expression of taste-related genes (bitter taste receptors; umami receptors; sweet taste receptors; effector proteins of the taste pathway).
ii) To investigate and compare taste chemosensory pathway responses to different taste compounds, in the CP.
iii) To assess the regulation of taste-like transduction pathway in CP by sex hormones.

In the long term, the scientific achievements attained with this project will contribute to better understand the mechanisms underlying the role of CP in the surveillance of the CSF and blood composition for the maintenance of brain homeostasis.

Chapter III

Tasting the cerebrospinal fluid: another function of the choroid plexus

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‘‘TASTING” THE CEREBROSPINAL FLUID: ANOTHER FUNCTION OF THE CHOROID PLEXUS?

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Abstract—The choroid plexus (CP) located in brain ventricles, by forming the interface between the blood and the cerebrospinal fluid (CSF) is in a privileged position to monitor the composition of these body fluids. Yet, the mechanisms involved in this surveillance system remain to be identified. The taste transduction pathway senses some types of molecules, thereby evaluating the chemical content of fluids, not only in the oral cavity but also in other tissues throughout the body, such as some cell types of the airways, the gastrointestinal tract, testis and skin. Therefore, we hypothesized that the taste transduction pathway could also be operating in the CP to assess the composition of the CSF. We found transcripts for some taste receptors (*Tas1r1*, *Tas1r2*, *Tas1r3*, *Tas2r109* and *Tas2r144*) and for downstream signaling molecules (α-*Gustducin*, *Plcβ2*, *ItpR3* and *TrpM5*) that encode this pathway, and confirmed the expression of the corresponding proteins in Wistar rat CP explants and in the CP epithelial cells (CPEC). The functionality of the T2R receptor expressed in CP cells was assessed by calcium imaging, of CPEC stimulated with the bitter compound D-Salicin, which elicited a rise in the intracellular Ca^{2+}. This effect was diminished in the presence of the bitter receptor blocker Probenecid. In summary, we described the expression of the taste-related components involved in the transduction signaling cascade in CP. Taken together, our results suggest that the taste transduction pathway in CPEC makes use of T2R receptors in the chemical surveillance of the CSF composition, in particular to sense bitter noxious compounds.

Key words: choroid plexus, taste transduction pathway, bitter taste receptor, blood–CSF barrier.

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Abbreviations: CNS, central nervous system; CP, choroid plexus; CPEC, CP epithelial cells; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; GPCRs, G protein-coupled receptors; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; IP3, inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; Plcβ2, phospholipase C-beta 2; PROP, 6-propyl-3-thiouracil; PTC, *N*-phenylthiourea; RT, room temperature; T1R1, taste receptors type 1; T2R2, taste receptors type 2; TBS, Tris-buffered saline; TrpM5, transient receptor potential channel M5; TTR, transthyretin; WGA, Wheat Germ Agglutinin.

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INTRODUCTION

The choroid plexus (CP), the main site of cerebrospinal fluid (CSF) production, is a highly vascularized branched structure that protrudes into each ventricle of the brain (Cserr, 1971; Keep and Jones, 1990; Del Bigio, 1995). The CSF fills the brain ventricles, the cisternal and subarachnoid space, covers the spinal cord, connects with paravascular spaces and, therefore is in close contact with brain parenchyma. CP epithelial cells (CPEC) constitute the blood–CSF barrier (Brightman and Reese, 1969; Pardridge, 2012) and synthesize and secrete a wide range of compounds determining their bioavailability in the central nervous system (CNS). Blood and brain-born molecules, in both sides of the barrier, can alter the CP secretome and modulate CSF composition accordingly. Moreover, the CP monitors the CSF for the presence of noxious compounds and protects the brain against neurotoxic insults using a complex detoxification system of the CSF (Strazielle and Ghersi-Egea, 2000; Gao and Meier, 2001; Emerich et al., 2005; Zappaterra and Lehtinen, 2012; Lehtinen et al., 2013; Bill and Korzh, 2014; Saunders et al., 2015) suggesting the existence of mechanisms to assess their composition.

The taste transduction pathway, originally identified in the tongue, senses different types of molecules, thereby evaluating the nutritive content of food and preventing the ingestion of toxic substances. Binding of tastant substances to specific receptors, in the taste buds, initiates signaling pathways leading to taste perception described as sweet, sour, bitter, salty, and umami. Sweet taste identifies energy-rich nutrients like sugars and carbohydrates, umami taste allows the recognition of amino acids, salty taste is generated mainly by Na^+ and ensures the proper dietary electrolyte balance, sour taste is potentiated by acids and together with the bitter taste detection they warn against the intake of potentially noxious and/or poisonous chemicals (Chandrashekar et al., 2006; Chaudhari and Roper, 2010; Iwatsuki and Uneyama, 2012).

The sensory responses to sweet, umami and bitter are initiated by the binding of the tastant to receptors that belong to the superfamily of G protein-coupled receptors (GPCRs). Two classes of taste GPCRs have been identified: taste receptors type 1 (T1Rs) and taste receptors type 2 (T2Rs). The T1R class responds to sweet (T1R2/T1R3) and umami (T1R1/T1R3) forming heterodimeric complexes, and the T2Rs respond to bitter stimuli (Chandrashekar et al., 2006; Chaudhari and Roper, 2010). Despite the fact that each taste receptor

recognizes a specific taste molecule type (sweet, umami or bitter), in the taste buds of the oral cavity, the signaling cascade is similar: the tastant binds to the receptor, resulting in a conformational change and in the activation of a series of signal transducers such as the taste-specific heterotrimeric G-protein gustducin (formed by α-gustducin, Gβ3 and Gγ13 subunits), which activates a specific phospholipase C-beta 2 (Plcβ2) to produce inositol 1,4,5-trisphosphate (IP3). The IP3 opens receptor type 3 ion channels (IP3R3), triggering an increase in intracellular Ca^{2+} levels which will activate a taste-selective cation channel, the transient receptor potential channel M5 (TrpM5), that eventually depolarizes the cell (Chandrashekar et al., 2006; Kinnamon, 2009; Chaudhari and Roper, 2010; Iwatsuki and Uneyama, 2012). Salt and sour detection, on the other hand, results in the direct opening of ion channels (Chaudhari and Roper, 2010).

Taste receptors were originally discovered in taste buds. However, an increasing number of studies have detected taste receptor expression and downstream signaling molecules in non-oral tissues, such as some cell types of the airways (Tizzano et al., 2011; Kinnamon, 2012; Krasteva and Kummer, 2012), in the gastrointestinal tract (Iwatsuki and Uneyama, 2012; Depoortere, 2014), in testis (Li, 2013; Xu et al., 2013), in the kidney (Liu et al., 2015), in keratinocytes (Wolfle et al., 2015) and in the thyroid gland (Clark et al., 2015). Another study revealed that T1R2 and T1R3 and their associated G-proteins (Gustducin) are expressed in the mammalian brain, particularly in the hypothalamus, hippocampus and cortex, suggesting that they work as a brain glucosensor (Ren et al., 2009). Furthermore, T2Rs were reported in the CNS, with a possible role in detecting bitter compounds (Singh et al., 2011b). In extraoral tissues the activation of taste chemosensory pathway has different roles, some of them yet to be clarified (Finger and Kinnamon, 2011). In respect to the respiratory tract, the inhalation of a toxic substance activates the bitter-taste receptors (T2Rs) provoking a reflex change in mice respiration (Tizzano et al., 2010); in human cultured airway epithelium T2R receptor activation leads to an increase in the frequency of ciliary beat (Shah et al., 2009). Moreover, the T2R receptor activation in the gut delays gastric emptying in rodents (Glendinning et al., 2008), which may help rodents to limit the intake of potentially toxic foods and increase elimination of toxins from the gut epithelium before they enter the circulation (Jeon et al., 2011). In the colon, activation of bitter receptors promotes fluid secretion by the epithelium to flush the noxious irritants out (Kaji et al., 2009). In spermatozoa T2R receptors may be responsible in sensing and avoiding toxins secreted by bacteria present in the female reproductive tract. However the functions of taste perception in the male reproductive system remain unclear (Li, 2013). In kidney cells, the expression of bitter receptor T2R105 is crucial in maintaining the structure of the glomerulus and renal tubule (Liu et al., 2015). The bitter signaling cascade activation in a keratinocyte cell line appears to influence cell differentiation (Wolfle et al., 2015) and in the thyroid gland changes its function and T3/T4 production possibly due to mediating a protective response to over ingestion of toxic compounds (Clark et al., 2015). All these tissues where the taste transduction machinery is active, work as barriers, and it seems that the taste system behaves as a sensor to assess the composition of body fluids.

We recently found expression of taste transduction-related genes in the rat CP (Quintela et al., 2013) within the frame of a cDNA microarray analysis. Moreover, *in situ* hybridization assays, performed by Ren et al. (2009), also identified *Tas1r2* and *Tas1r3* gene expression in the epithelial cells of the CP. So, we hypothesized that the taste-like chemosensory pathway may be used by the epithelial cells of the CP, enabling an accurate detection of the CSF chemical and nutritional composition, thereby inducing cellular responses according to the CNS physiological state.

Therefore, the aim of the present work was to analyze the expression of the taste transduction machinery in the CP, and to examine its functioning, as it may be particularly relevant for the monitoring the CSF composition.

EXPERIMENTAL PROCEDURES

Animals

Wistar rats were handled in compliance with the NIH guidelines, and the European Union rules for the care and handling of laboratory animals (Directive 2010/63/EU). CPs were collected from the lateral ventricles, from animals euthanized under administration of ketamine/xylazine anesthesia. Two-month-old animals were used for preparing CP paraffin sections and for whole-mount immunohistochemistry analysis. Primary cultures of CPEC were obtained from 3 to 5-day-old newborn rats CP. RNA and protein extracts were prepared from 2 to 3-month-old rat CPs.

Primary cultures of rat CPEC

Primary cultures of CPEC were established and maintained as previously described (Quintela et al., 2008). Briefly, dissected CPs were mechanically and enzymatically digested in phosphate-buffered saline (PBS) containing 0.2% pronase (Fluka, Seelze, Germany) at 37 °C for 5 min. Dissociated cells were washed in Dulbecco's modified Eagle medium – high glucose (DMEM, Sigma, UK) with 10% fetal bovine serum (FBS, Biochrom AG, Berlin) and 100 units/mL of penicillin/streptomycin (Sigma, UK). Cells were seeded into 12 mm wells (3CPs/well) for immunocytochemistry, and in μ-Slide 2-well ibiTreat chambers (Ibidi, Germany) for intracellular calcium analysis, and cultured in DMEM supplemented with 100 units/mL antibiotics, 10% FBS, 10 ng/mL epidermal growth factor (Sigma, UK), 5 μg/mL insulin (Sigma, UK) and 20 μM cytosine arabinoside (Sigma, UK), in a humidified incubator in 95% air with 5% CO_2 at 37 °C. The growth medium was replaced 1 day after the initial seeding, and every 2 days thereafter until they were differentiated.

Reverse transcription PCR

Total RNA was extracted from a pool of six CPs with TripleXtractor (Grisp, Portugal) following the manufacturer's instructions. RNA was quantified by spectrophotometry at 260 nm (NanoPhotometer, IMPLEN, München, Germany), and its integrity was assessed by agarose gel (1%) electrophoresis. A total of 1 μg of RNA was reverse transcribed with the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., USA), according to the manufacturer's instructions. PCR reactions were carried out in a final volume of 20 μL with the KAPA2G Fast ReadyMix PCR Kit (KapaBiosystems, UK) and 0.3 μM of each primer (Table 1). PCR protocols consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 60–64 °C (Table 1) for 10 s and extension at 72 °C for 1 min. PCR products were analyzed in a 1.5% agarose gel, visualized under UV with green safe 1% staining (Nzytech, Portugal), and Sanger sequenced (STABvida, Portugal) to confirm the gene identity.

Total protein extraction and Western blot analysis

CPs were homogenized in RIPA buffer (NaCl 150 mM, Sodium deoxycholate 0.5%, SDS 0.1%, Triton X-100 1%, Tris 50 mM pH 8.0, PMSF 1 mM and 10 μL/mL Complete EDTA-Free protease inhibitor cocktail, Roche, Portugal). Suspensions were centrifuged at 10,000×*g* for 10 min at 4 °C and the pellets discarded. Total protein measurement was performed with Pierce BCA Protein Assay Kit (23227) following the manufacturer's instructions. An amount of 30 μg of total protein was mixed with loading buffer containing 3% β-mercaptoethanol, boiled for 10 min, and then loaded in a 12% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (GE Healthcare, UK), which was blocked with 1% nonfat dry milk, in Tris-buffered saline (TBS), for 1 h at room temperature (RT). After incubation with the primary antibodies against T1R1, T1R2, Gustducin, Plcβ2 and TrpM5 (Table 2), membranes were rinsed three times with 0.1 M TBS containing 0.01% Tween 20 and incubated with the respective secondary antibody HRP linked for 1 h at RT (Table 2). Signal detection was performed with Clarity™ Western ECL Substrate (Bio-Rad, USA) and images were acquired with the Image Lab software in a ChemiDoc™ MP (Bio-Rad, USA). These experiments were done for three different animals. The specificity of primary antibodies was assessed with a pre-incubation of the respective peptide, overnight at 4°C according to manufacturer's instructions, and followed by the protocol above.

Immunohistochemistry

Paraffin-embedded CP slices were processed as follows: incubation twice in xylene for 5 min, 100% ethanol for 3 min twice, 95% ethanol for 3 min, 80% ethanol for 3 min, running tap water for 5–10 min. Endogenous peroxidase activity was blocked with H_2O_2 3% for 10 min at RT. After blocking with 1% bovine serum albumin in PBS at RT for 1 h, slices were washed with PBS containing 0.1% Tween 20 (PBS-T) and incubated with the primary antibody (T1R1, T1R2, Gustducin, Plcβ2 or TrpM5) (Table 2), overnight at 4 °C. After a three-time wash step with PBS-T, slices were incubated with biotinylated secondary antibodies (DAKO, USA) for 1 h at RT, followed by incubation for 30 min with the streptavidin peroxidase (DAKO, USA). Immunoreactivity was detected with diaminobenzidine peroxidase (DAB 50x Dako REAL, Dako, USA). Following DAB reaction,

Table 1. Primer sequences and RT-PCR annealing temperatures (AT)

Gene name	Gene symbol	GeneID	Sequence primer 5′–3′	Size (bp)	AT (°C)
Taste receptor, type 1, member 1	*Tas1r1*	29407	Fw – CTGTTCTCCCTCCATGGTGAC Rv – GTCGTACAGCTCATACCCCAG	186	60
Taste receptor, type 1, member 2	*Tas2r2*	100270683	Fw – CCTCCATGCCAACGTGAAGA Rv – CATCCACCATCTCGTAGCCG	182	60
Taste receptor, type 1, member 3	*Tas1r3*	170634	Fw – GCTATGACTGTGTGGACTGC Rv – TGCGAGGTAAGCAGGTTGTG	120	60
Taste receptor, type 2, member 109	*Tas2r109*	690572	Fw – TCACCACCTTAGCAACCTCC Rv – CCACAGATTACTGGCGACCT	125	64
Taste receptor, type 2, member 144	*Tas2r144*	500101	Fw – CGCGTGGCTCAATGTCTTCT Rv – GAGCCGAGGCATCAACACTAC	100	60
Guanine nucleotide-binding protein, alpha transducing 3 (α-Gustducin)	*Gnat3*	286924	Fw – AGCAGTTCAACCCCTTCTCG Rv – GCAGTAGGTGACAGCTTGGT	141	62
Guanine nucleotide-binding protein (G protein), beta polypeptide 3	*Gnb3*	60449	Fw – ATGTGAGGGAAGGGACCTGT Rv – CGTGATGCCACAGATGATGC	188	60
Phospholipase C, beta 2	*Plcb2*	85240	Fw – AGCAGTTCAACCCCTTCTCG Rv – CGTGATGCCACAGATGATGC	181	60
Inositol 1,4,5-trisphosphate receptor, type 3	*Itpr3*	25679	Fw – CGAGGTGGAAACCTTCGTGA Rv – AAATCAGCTCCTGGGTGACG	119	60
Transient receptor potential cation channel, subfamily M, member 5	*Trpm5*	365391	Fw – ATTTTCCGCCGTGTGCTCTA Rv – CAACGGGTGAAGAGAGCAGT	102	60
Sodium channel, non-voltage-gated 1, gamma subunit	*Scnn1g*	24768	Fw – CACTGGAGCCAAGGTGCTTA Rv – ACTGGCTGTAAGGTTCGCTC	143	60

Fw – forward; Rv – reverse; bp – base pair.

tissue sections were counterstained with hematoxylin. Negative control slices were treated under the same conditions without primary antibody. After dehydration and embedding, the slices were mounted and the images were acquired in an Axio Imager A1 microscope, with an AxioVision camera and software (Carl Zeiss, Germany) using a magnification of 100×. Immunohistochemistry (IHC) staining was performed with slices from CPs collected from three different animals.

Immunofluorescence

The whole-mount immunofluorescence (IF) in CP explants (the complete structure) from newborn rats were used for taste-related protein localization. CPs were collected, one CP per well, to a 96-well plate, containing a 4% PFA (Merck, Portugal) solution and after 30 min, replaced by a 30% sacarose solution (Sigma, UK) until CPs settled at the bottom. For membrane staining, incubation with 5 μg/mL Wheat Germ Agglutinin (WGA) Alexa Fluor® 594 conjugate (Molecular Probes®) was performed, for 20 min. After three washes with PBS, CPs were submerged in the blocking solution (PBS with BSA 2.5% and Triton X100 0.1%) for 8 h at 4 °C. Subsequently, the specific primary antibody diluted in the blocking solution was applied overnight at 4 °C (Table 2). CPs were washed six times in PBS-T and incubated with the appropriate secondary antibody, for 3 h. For double labeling, first and second antibodies were applied sequentially with a wash step in between. Nuclei were stained with Hoechst 33342, diluted 1:1000 in PBS, for 20 min. After the final washing step, CPs were placed on glass slides and covered with a lamella using the mounting medium Entellan (Merck, Portugal). Confocal images were obtained with a LSM710 confocal laser scanning microscope (Carl Zeiss, Germany) under a 63× magnification. These experiments were done in triplicate.

For immunocytochemistry (ICC), primary cultures of CPEC were grown in 12-well plates with glass coverslips. Cells were fixed with 4% PFA (Merck, Portugal) in PBS for 10 min. After washing with PBS, cells were incubated with 0.1% Triton X-100 in PBS for 5 min and then in blocking buffer PBS-T and FBS 20%, for 1 h at RT. Subsequently, cells were incubated overnight at 4 °C with the primary antibodies for T1R1, T1R2, α-Gustducin, Plcβ2 and TRPM5 (Table 2). Cells were washed in PBS-T six times and incubated with the appropriate secondary fluorescent antibody. For double staining CPEC were grown in 12-well plates with glass coverslips. Cells were fixed with methanol/acetone (1:1) for 10 min at −20 °C. After washing with PBS, cells were incubated with 0.1% Triton X-100 in PBS for 5 min and then in blocking buffer containing 0.2% gelatin in PBS-T, for 1 h at RT. Subsequently, cells were incubated overnight at 4 °C with the primary antibodies for T1R3, α-Gustducin, Plcβ2 and TRPM5 (Table 2). Cells were washed in PBS-T six times and incubated with the appropriate secondary fluorescent antibodies. Nuclei were stained with Hoechst 33342 for 5 min. After the final washing step, coverslips were placed on glass slides using the mounting medium Entellan (Merck, Portugal). Cell preparations were examined on a LSM710 confocal laser scanning microscope (Carl Zeiss, Germany) at a 63× magnification.

The specificity of primary antibodies T1R1, T1R2, α-Gustducin, Plcβ2 and TRPM5 was performed with a pre-incubation with the respective peptide (Table 2), overnight at 4 °C, followed by the above protocol. Cross-reactions of secondary antibodies between each other or the tissue studied was evaluated with incubation of CPEC and explants with the secondary antibodies only.

Single-cell calcium imaging

Changes in intracellular calcium levels were measured in primary cultures of CPEC with Fura-2 AM (Molecular Probes, USA). CPEC were incubated for 1 h at 37 °C with calcium-free Tyrode's solution (Tyrode's solution: NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 2 mM, Na-pyruvate 10 mM, glucose 10 mM, HEPES 10 mM, $NaHCO_3$ 5 mM, pH 7.4), loaded with 5 μM Fura-2 AM 0.1% BSA and 0.02% pluronic acid F-127 in Tyrode's, washed and loaded in Tyrode's for 30 min before acquisition. The μ-Slide 2-well ibiTreat chambers (Ibidi,

Table 2. List of the antibodies used for Western blot (WB), immunohistochemistry (IHC) and immunofluorescence (IF) analyses

	Blocking peptide	Host	WB	IH	IF	Company	Ref.
Primary anybody							
T1R1	Yes	Rabbit	1:250	1:100	1:100	Santa Cruz Biotechnology, Inc.	sc-50308
T1R2	Yes	Rabbit	1:250	1:100	1:100	Santa Cruz Biotechnology, Inc.	sc-50306
T1R3	–	Goat	–	–	1:100	Santa Cruz Biotechnology, Inc.	sc-22458
T2R144	–	Goat	1:250	–	1:100	Santa Cruz Biotechnology, Inc.	sc-165636
α-Gustducin	Yes	Rabbit	1:250	1:100	1:100	Santa Cruz Biotechnology, Inc.	sc-395
PLCβ2	Yes	Rabbit	1:250	1:100	1:100	Santa Cruz Biotechnology, Inc.	sc-206
TRPM5	Yes	Rabbit	1:500	1:300	1:400	Abcam	AB104566
TTR	–	Rabbit	–	–	1:200	Dako	A0002
Secondary antibody							
Alexa Fluor® 488 Anti-Rabbit		Goat	–	–	1:1000	Molecular Probes®	A-11008
Alexa Fluor® 488 Anti-Goat		Donkey	–	–	1:1000	Molecular Probes®	A-11055
Alexa Fluor® 546 Anti-Goat		Donkey	–	–	1:1000	Molecular Probes®	A-11056
Biotinylated Secondary Antibodies		–	–	–	–	DAKO	K5001
Anti-rabbit IgG-HRP		Goat	1:50000	–	–	Santa Cruz Biotechnology, Inc	sc-2004
Anti-goat IgG-HRP		Chicken	1:50000	–	–	Santa Cruz Biotechnology, Inc	sc-2953

Germany) were placed on an inverted fluorescence microscope (Axio Imager A1, Carl Zeiss, Germany). The bitter agonists Denatonium benzoate, D-Salicin, 6-propyl-3-thiouracil (PROP) and *N*-phenylthiourea (PTC) (Sigma, UK) were dissolved in Tyrode's at 100 mM. Before each stimulus an experiment using only vehicle (Tyrode's) has been done, during 5 min (baseline) and no alterations were observed. CPEC were then stimulated at 10 mM for 3 min, with each compound. The bitter taste receptor blocker Probenecid (Sigma, UK) was dissolved in 1 N NaOH at 0.17 M and diluted in Tyrode's at 1 mM (working solution). Stimuli at 1, 5, 10, 15 and 20 mM D-Salicin were carried out with or without Probenecid, for 3 min. For that, CPECs were incubated with Probenecid 30 min prior to application of D-Salicin at RT. The intracellular calcium concentration was evaluated by quantifying the ratio of the fluorescence emitted at 520 nm following alternate excitation at 340 nm and 380 nm, using a Lambda DG4 apparatus (Sutter Instruments, Novato, CA, USA) and a 520-nm bandpass filter (Carl Zeiss, Germany) under a 40× objective (Carl Zeiss, Germany) with an AxioVision camera and software (Carl Zeiss, Germany). Data were processed using the Fiji software (MediaWiki, USA and Germany). Results are presented as an average of the changes in intracellular calcium levels of 15–20 cells from three or more independent experiments.

Data analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered for p values <0.05 using *t*-student test and One-Way analysis of variance (ANOVA) followed by Tukey test.

RESULTS

The taste-related machinery is expressed in rat CP

To understand whether the CP can detect and respond to CSF-occurring compounds, through activation of the taste transduction machinery, we set out to examine the presence of gene transcripts belonging to the two classes of taste GPCRs (*Tas1r1*, *Tas1r2*, *Tas1r3*, *Tas2r109* and *Tas2r144*), and some of the downstream signaling molecules (*Gnat3*, *Plcb2*, *Itpr3*, *Trpm5* and *Scnn1g*). To this end, total RNA isolated from adult rat CPs, was used in RT-PCR with specific primers (Table 1), matching exon–exon junctions, and the length of the PCR products corresponded to the expected size for all studied genes (Fig. 1). The inexistence of bands in the control samples, performed without reverse transcriptase, confirmed the absence of genomic DNA contamination. Yet, to verify the primers specificity, all the PCR products were Sanger sequenced and the results revealed an identity higher than 97% to the published cDNA sequences. All these analysis were also confirmed using total RNA isolated from primary cultures of rat CPEC (data not shown).

The expression and characterization of taste transduction proteins, were studied in total protein extracts, prepared from rat CPs, and Western blot assays were done with the available antibodies for taste receptors and for some of the downstream taste transduction proteins. Positive protein expression of the expected molecular weights was obtained for: T1R1 (93 kDa), T1R2 (96 kDa), T2R144 (80 kDa), α-Gustducin (100 kDa), Plcβ2 (130 kDa) and TrpM5 (140 kDa) (Fig. 2). Data are representative for three rats from three independent experiments.

Taste-related proteins are localized in the epithelial cells of rat CP

To further explore the localization of taste-related proteins in rat CP, we carried out IHC and IF analyses. More specifically, to determine the tissue localization of taste-related proteins, we chose five taste-signaling molecules (T1R1, T1R2, α-Gustducin, Plcβ2 and TrpM5) and, as seen in Fig. 3, positive immunostaining was observed in the epithelial cells of rat CP. Furthermore, their expression in the CPEC was confirmed by double-labeling IF analysis using different host antibodies against taste receptors (T1R3 and T2R144), and the CPEC marker transthyretin (TTR) (Mollgard et al., 1979) in whole-mount explants. The results, seen in the merged column of Fig. 4a, showed co-expression of each of the taste receptors T1R3 and T2R144 with TTR, in the epithelial cells of the CP.

As umami and sweet receptors are described as forming heterodimers, we also performed the co-expression analysis of taste proteins that encode the umami (T1R1/T1R3) and the sweet (T1R2/T1R3) receptors. As seen in Fig. 4b, double labeling with T1R1 and T1R3 antibodies, revealed co-expression of these two proteins in the epithelial cells of the CP, as well as with double labeled T1R2 and T1R3 antibodies.

Primary antibody specificity was examined by the blockage with the respective peptide (Table 2), before the antibody incubation, showing that the antibodies used are specific for their targets. The secondary antibody control revealed no fluorescence, indicating that they do not cross-react with each other or with CP explants (data not shown).

Taste-related proteins are localized in the cytoplasm and in the plasma membrane of epithelial CP cells

To analyze cell localization of taste-related proteins we used IF in whole-mount rat CP explants. With that purpose, CPs were double labeled with antibodies against each of the taste-related proteins (T1R1, T1R2, T1R3, T2R144, α-Gustducin, Plcβ2 and TrpM5) and the fluorescent marker of glycosylated surface-expressed proteins, WGA. In single-channel confocal images (Fig. 5, Alexa 488 column) it was possible to observe a cytoplasmatic expression of taste-related proteins in CPEC. Co-localization of each protein studied and the WGA immune reactivities to the plasma membrane (Fig. 5, Merged + Hoechst column), indicated that each protein studied indeed localized to the plasma membrane of the epithelial cells of rat CP.

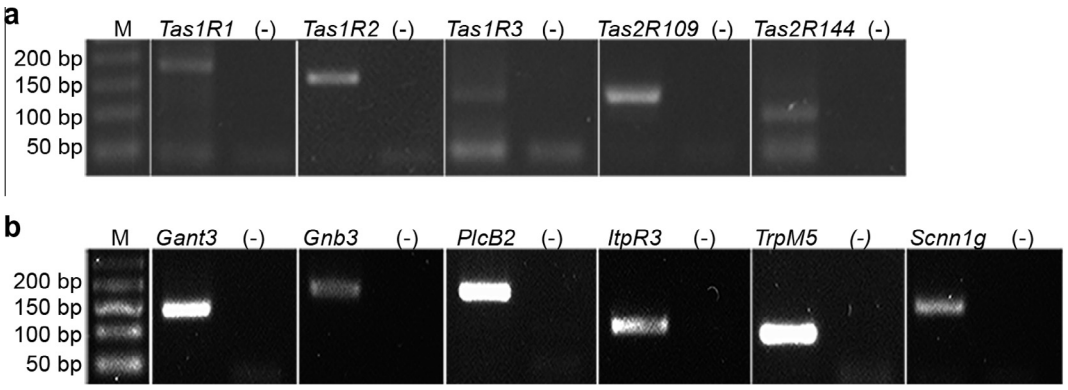

Fig. 1. Taste-related genes are expressed in rat CP. Gel electrophoresis of RT-PCR products and staining with green safe demonstrates expression of taste receptor genes in rat CP (a) and downstream signaling molecules (b). M: molecular weight marker (GRS Ladder 50 bp, Grisp); (−): negative control.

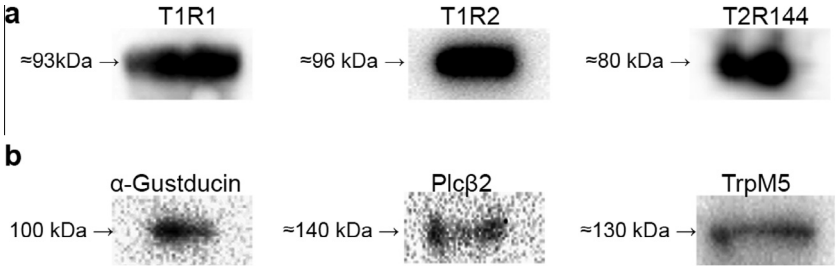

Fig. 2. Western blot results illustrating the expression of taste receptor proteins (≈93 kDa, T1R1; ≈96 kDa, T1R2; ≈80 kDa, T2R144) (a) and taste transduction proteins (≈100 kDa, α-Gustducin; ≈140 kDa, Plcβ2; ≈130 kDa, TrpM5) (b), in rat CP.

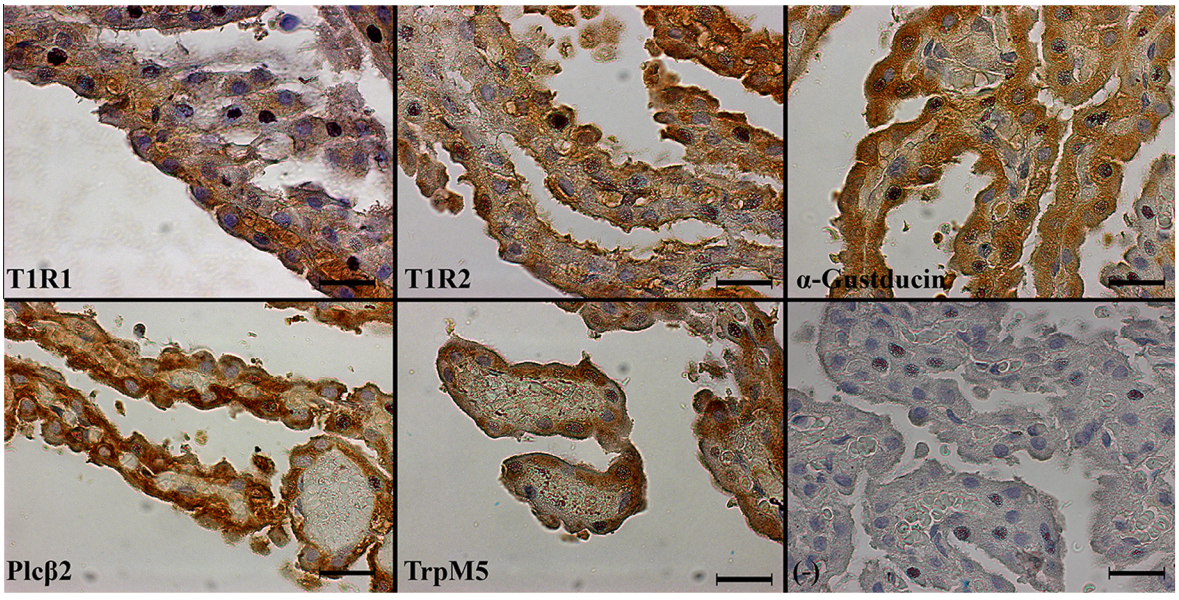

Fig. 3. Representative images showing the immunolocalization of taste transduction proteins in rat CP slices. T1R1, T1R2, α-Gustducin, Plcβ2, TrpM5 and negative staining (−). Immunohistochemistry was performed using DAB and hemotoxylin (scale bars = 20 μm).

The expression of taste-related proteins in the epithelial cells of the CP was also evaluated by ICC in primary cultures of CPEC and is shown in Fig. 6, immunodetection was observed for all the taste-related proteins analyzed: T1R1, T1R2, T1R3, T2R144, α-Gustducin, Plcβ2 and TrpM5. Co-expression of the taste receptor T1R3 and the downstream signaling proteins α-Gustducin, Plcβ2 and TrpM5 involved in the taste transduction pathway were analyzed and, as observed in Fig. 7 the CPEC that expressed T1R3 are also

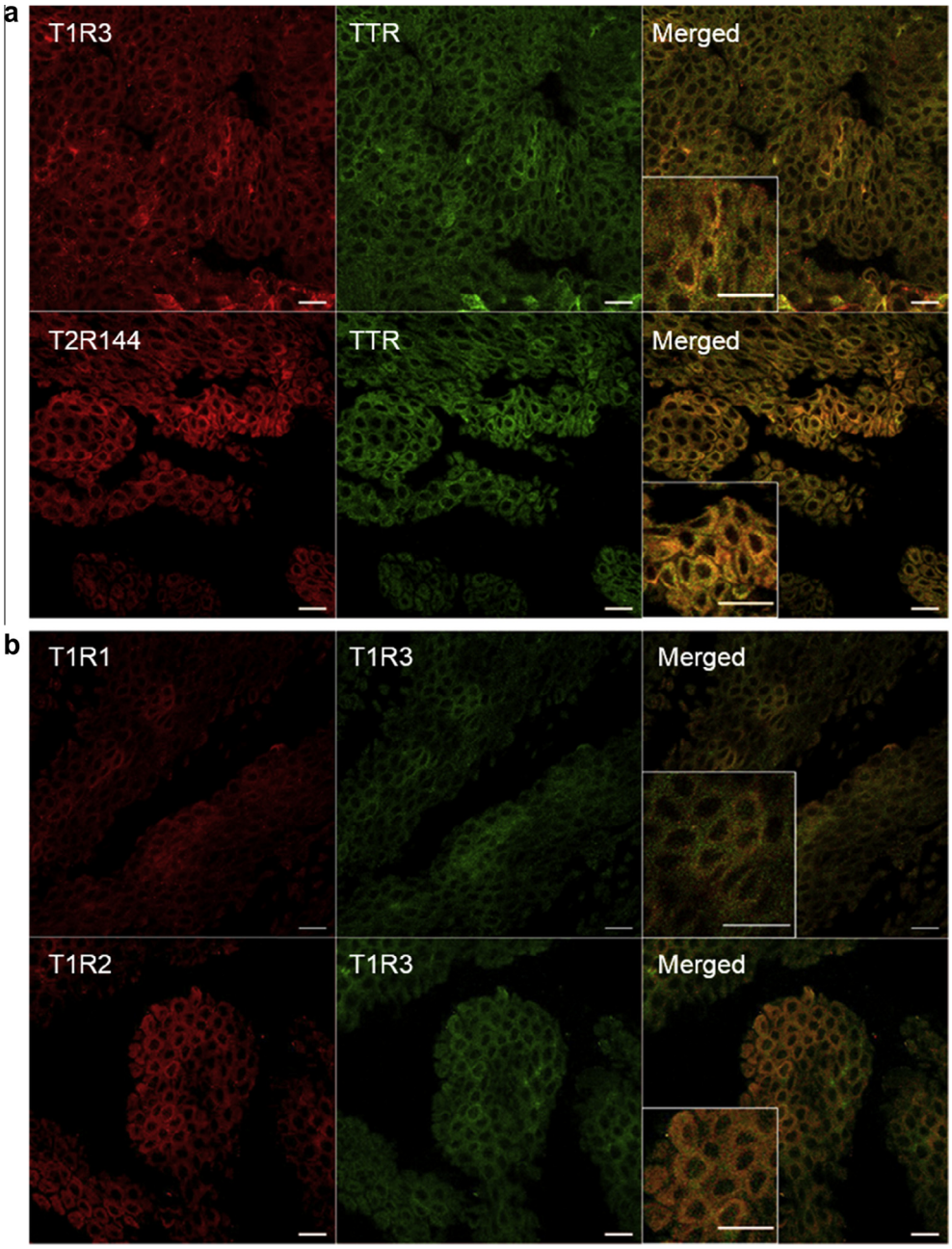

Fig. 4. Confocal images of taste-related protein expression in epithelial cells of rat CP explants. (a) Co-expression of taste receptors T1R3 or T2R144 (red) with the CP epithelial cell marker, TTR (green), in rat CP explants by double immuno-labeling, demonstrating taste receptor expression in the epithelial cells of rat CP. (b) Double immunodetection of the heterodimeric taste receptors in rat CP explants, demonstrating co-expression of: umami subunits receptors T1R1/T1R3 (red/green) and sweet subunit receptors T1R2/T1R3 (red/green) (scale bars = 20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

positive for the downstream elements studied. Co-localization can be seen by the appearance of yellow/orange fluorescence in the merge column and we may conclude that all cells were positive for the receptor T1R3 and each of the downstream signaling proteins (α-Gustaducin, Plcβ2 and TrpM5).

The analysis of antibody specificity was performed by the blockage with the respective peptide, when available (Table 2), before the antibody incubation. The results obtained reveal that the antibodies used are specific for their targets (data not shown).

A bitter taste compound elicits calcium responses in CPEC in a dose-dependent manner

Having established that the taste-related machinery is reliably expressed in CPEC, we proceeded with their functional analysis by measuring intracellular Ca^{2+} response in CPEC primary cultures, using increasing concentrations of a taste stimulus. To assess the function of the T2R receptor expressed in CP cells, we first analyzed the effect of four bitter compounds (Denatonium benzoate, D-Salicin, PROP and PTC)

because these compounds are potentially toxic, and may exist in the CSF, either as natural occurring compounds, potential contaminants or drugs. Only D-Salicin elicited a response, monitored by measuring the intracellular Ca^{2+} of primary cultures of CPEC, with single-calcium imaging of individual cells after a 5-min baseline (with the vehicle) without variation in Ca^{2+} levels.

The results obtained from increasing concentrations of D-Salicin (1, 5, 10, 15 and 20 mM) are presented in Fig. 8, showing that D-Salicin elicited a dose-related response, with statistical significance ($p < 0.0001$). The lower concentration of 1 mM corresponded to the lower calcium intake ($\Delta F = 0.256858 \pm 0.017144$) by the cell while the higher concentration of 20 mM corresponded to the major intake of calcium ($\Delta F = 0.404041 \pm 0.010333$).

The response of CPEC to a bitter stimulus can be suppressed by a bitter receptor blocker

To analyze if the D-Salicin response occurred via bitter receptor, experiments were performed with the blockage of T2Rs with Probenecid (Greene et al., 2011). Blockage of T2Rs, with 1 mM Probenecid, reduced significantly the calcium intake after D-Salicin stimulus with 1 mM ($\Delta F = 0.172957 \pm 0.022615$, $p = 0.001$), 5 mM ($\Delta F = 0.214733 \pm 0.036667$, $p = 0.0006$) and 10 mM ($\Delta F = 0.270333 \pm 0.016425$, $p = 0.0004$) when compared with the calcium intake without blockage ($\Delta F = 0.256858 \pm 0.017144$, $\Delta F = 0.325482 \pm 0.019934$ and $\Delta F = 0.358934 \pm 0.025838$, respectively) (Fig. 8). Our results showed that pre-treatment of cultured cells with the bitter receptor blocker Probenecid, at 1 mM, reduced calcium response by CPEC after a stimulus with D-Salicin using three different concentrations (1, 5 and 10 mM).

DISCUSSION

Lying at the interface between the blood and the CSF, the CP plays a pivotal role in monitoring CSF composition (Bill and Korzh, 2014). Yet, the mechanisms underlying the capacity of the CP to surveil the chemical composition of the CSF are poorly understood. Our recent discovery that some genes from the taste transduction pathway are up-regulated in CP of gonadectomized rats (Quintela et al., 2013), and the existence of sweet receptor complex *Tas1R2* and *Tas1R3* transcripts in CP described by Ren et al. (2009), led us to ask whether this transduction pathway is functional in CP. Considering the nongustatory tissues where the taste transduction pathway has been reported, like the gastrointestinal tract (Iwatsuki and Uneyama, 2012; Depoortere, 2014), the respiratory system (Tizzano et al., 2011; Kinnamon, 2012; Krasteva and Kummer, 2012), testis (Li, 2013; Xu et al., 2013), kidney (Liu et al., 2015), skin (Wolfle et al., 2015) and brain (Ren et al., 2009; Singh et al., 2011a), the study of taste transduction in CP may contribute to better elucidate the novel paradigm in sensory physiology where taste and odorant receptors may act as sensitive chemoreceptors (Foster et al., 2014), to surveil the composition of the CSF and brain interstitial fluid.

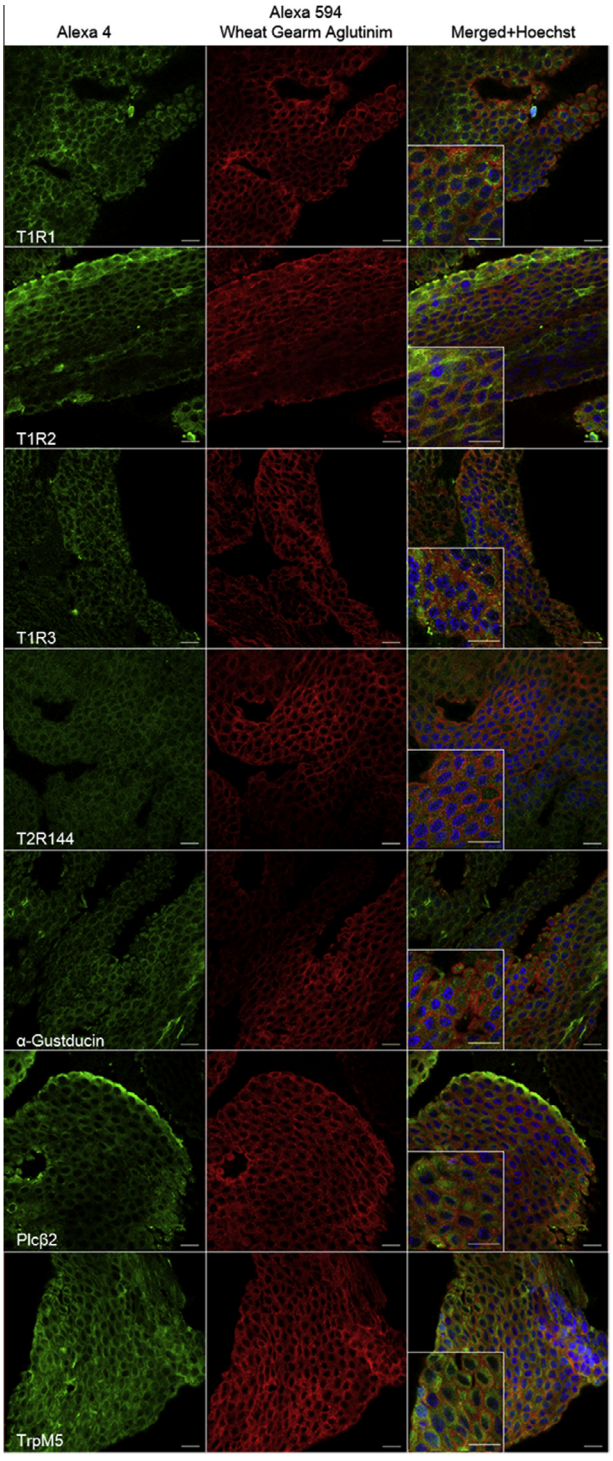

Fig. 5. Cellular localization of taste-related proteins in rat CP explants. Confocal immunofluorescence images: the Alexa 488 channel (green) represents expression of the protein of interest T1R1, T1R2, T1R30, T2R144, α-Gustducin, Plcβ2, and TrpM5 and the Alexa 594 (red) channel the membrane marker WGA. The merge column (yellow) is the overlap of the two channels and Hoechst (blue) (scale bars = 20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In the present study, we first demonstrate the presence of taste receptors and downstream signaling

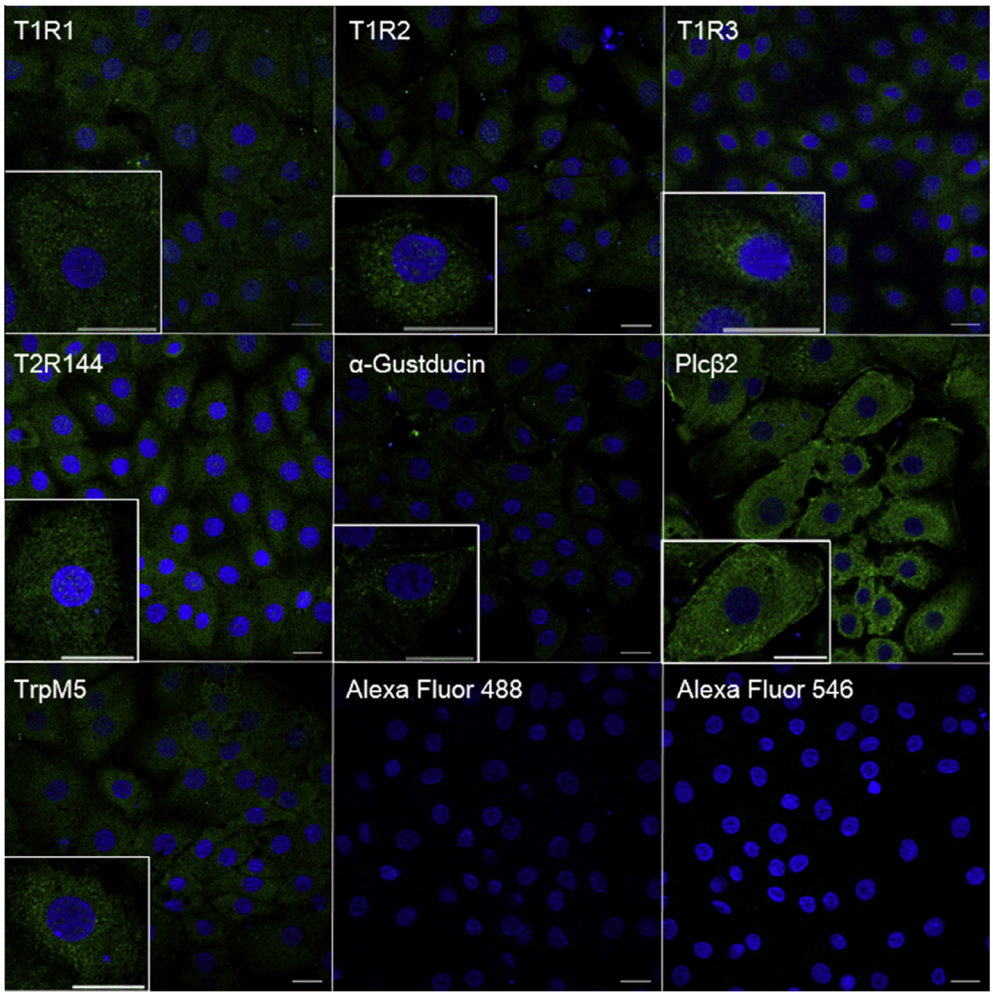

Fig. 6. Confocal immunofluorescence images of taste transduction-related proteins in primary cultures of choroid plexus epithelial cells (CPEC). Immunodetection of T1R1, T1R2, T1R3, T2R144, α-Gustducin, Plcβ2 and TrpM5 in CPEC (green), with nucleus labeled with Hoechst (blue). T1R1, T1R2, α-Gustducin, Plcβ2 and TrpM5 were labeled with the secondary antibody Alexa Fluor 488Anti-Rabbit and T1R3 and T2R144 with Alexa Fluor 546 Anti-Goat. Alexa Fluor 488 Anti-Rabbit and Alexa Fluor 546 Anti-Goat are controls without the primary antibody (scale bars = 20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

molecules of the taste transduction pathway, using RT-PCR, Western blot and IHC. All IHC and IF analyses were carried out, with validated antibodies, previously described not only in gustatory tissues but also in non-gustatory tissues (Miyoshi et al., 2001; Hass et al., 2007; Mace et al., 2007; Yang et al., 2007; Shigemura et al., 2009; Dehkordi et al., 2012; Li and Zhou, 2012; Li et al., 2013; Kendig et al., 2014; Clark et al., 2015; Liu et al., 2015), with the exception of the T2R144 (sc-165636) and T1R2 (sc-50603) antibodies.

We confirmed the presence of transcripts for taste receptors (*Tas1r1*, *Tas1r2*, *Tas1r3*, *Tas2r109* and *Tas2r144*) and downstream signaling molecules (*Gnat3*, *Plcb2*, *Itpr3*, *Trpm5* and *Scnn1g*) in the epithelial cells of rat CP by RT-PCR, corroborating data from our previous cDNA microarray study (Quintela et al., 2013). Protein extracts from CP were also analyzed, by Western blot, with six specific antibodies assessing different steps of the taste signaling cascade: tastant recognition – taste receptors T1R1, T1R2 and T2R144; mediators of the signal transduction cascade – α-Gustducin and the taste-specific phospholipase (Plcβ2); and a taste-selective cation channel TrpM5 that will depolarize the cell and promote the signal propagation (Chandrashekar et al., 2006; Chaudhari and Roper, 2010). Only α-Gustducin and T2R144 exhibited higher molecular weights than expected. A molecular weight of 100 kDa was obtained for α-Gustducin instead of 40 kDa, that has also been described in the small intestine (Mace et al., 2007). T2R144 showed double of its expected weight, 80 kDa instead of 40 kDa. These results point to the detection of protein dimers instead of the monomer isoform for the α-Gustducin and T2R144.

To confirm that expression of the taste transduction machinery was specific to epithelial cells of CP, double labeling of CP explants with two of the target proteins (T1R3 and T2R144) and TTR, a CPEC-specific marker (Herbert et al., 1990) was carried out confirming our expectations. In the mouse, it is well known that sensory responses to sweet, umami and bitter are initiated by the binding of the tastant to umami (T1R1/T1R3), sweet (T1R2/T1R3) and bitter (T2Rs) receptors (Lindemann,

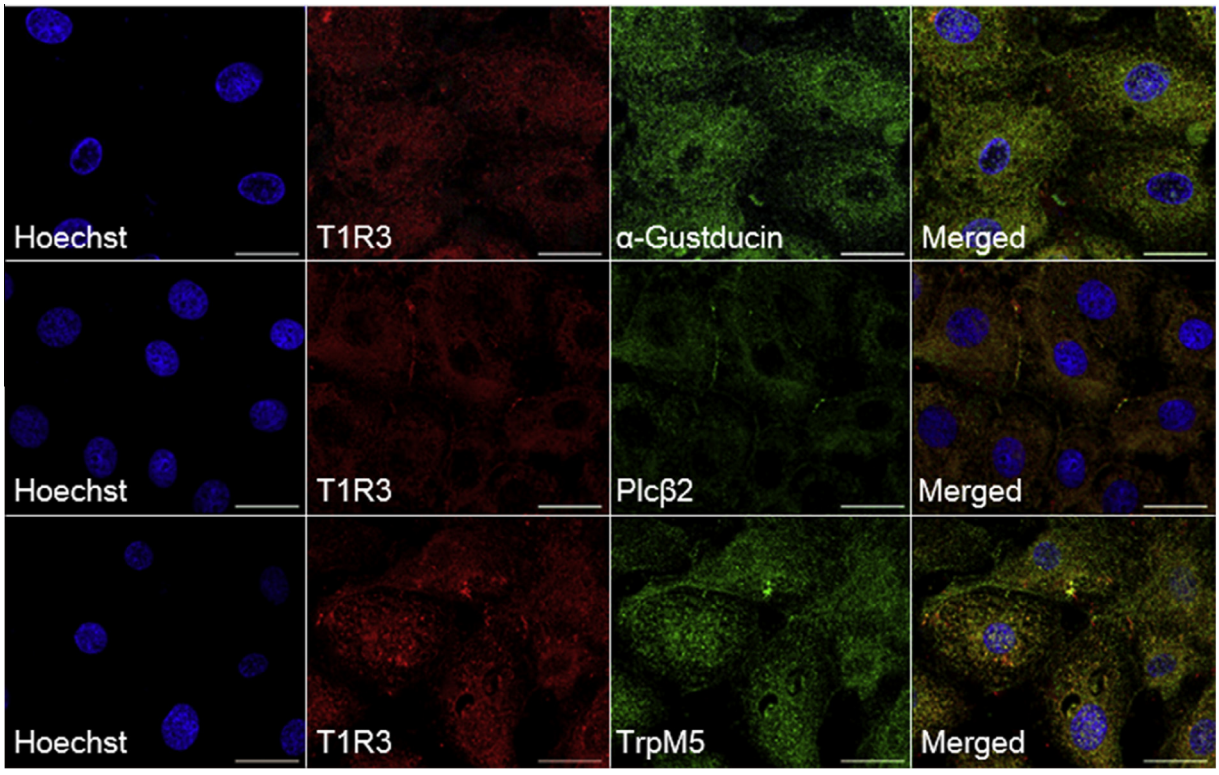

Fig. 7. Confocal immunofluorescence images demonstrating the co-expression of the taste receptor T1R3 (red) and the taste-related proteins α-Gustducin, Plcβ2 and TrpM5 (green) in primary cultures of choroid plexus epithelial cells (CPEC) (scale bars = 20 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

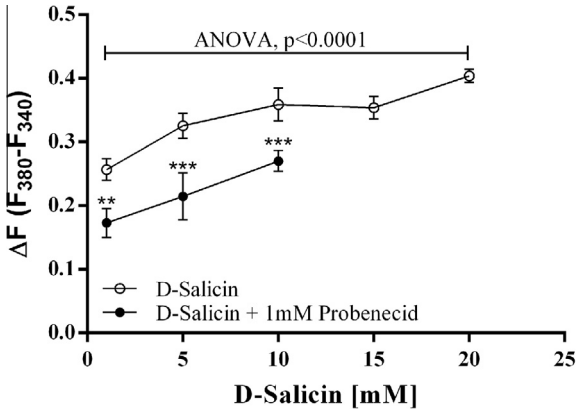

Fig. 8. Calcium responses of epithelial cells of rat CP were inhibited by a bitter receptor blocker (Probenecid). The increase in the intracellular Ca^{2+} concentration in response to several concentrations of D-Salicin (1, 5, 10, 15 and 20 mM) (ANOVA, $p < 0.0001$) in the absence (○) and in the presence of Probenecid (●) (*T* test, $^{**}p \leqslant 0.001$, $^{***}p \leqslant 0.0006$).

2001; Pydi et al., 2014). The double immuno staining of CP explants with T1R1/T1R3 or T1R2/T1R3 revealed co-expression of each of these heterodimers in the epithelial cells of the CP. The presence of key taste transduction signaling components such as taste receptors T1R1/T1R3, T1R2/T1R3 and T2R144, and downstream molecules, namely α-Gustducin, Plcβ2 and TrpM5 suggests that the taste transduction pathway may be one of the mechanisms enabling the CP to have a role in the surveillance of CSF composition (Mortazavi et al., 2014).

Considering that the taste transduction pathway is initiated by the recognition of the chemical by its cognate receptors described as being present in the cell membrane (Gilbertson et al., 2000), the cellular localization of taste-related proteins in the CP was analyzed. Double staining of WGA and the taste proteins (T1R1, T1R2, T1R3, T2R144, α-Gustducin, TrpM5 and Plcβ2) was carried out in CP explants revealing cytoplasmic and plasma membrane localizations of taste-related proteins.

Once established that all the machinery for chemical taste recognition was present in the CP, we asked if CP responds to taste stimuli and for that purpose, an *in vitro* model of epithelial cells of CP was used. Despite the fact that primary cultures of CPEC are a widely and well-described *in vitro* model used to study the response of CP to chemical compounds (Quintela et al., 2008, 2009), it was necessary to verify if primary cultures of CPEC expressed the proteins involved in the taste transduction pathway. Immunocytochemistry results confirmed the expression of T1R1, T1R2, T1R3, T2R144, Gustducin, Plcβ2 and TrpM5 in CPEC.

In the frame of CP's role in monitoring the CSF for the presence of noxious compounds and protecting the brain against neurotoxic insults (Bueno et al., 2014; Mortazavi et al., 2014) and because most of the toxic compounds correspond to bitter molecules (Behrens et al., 2009; Pydi et al., 2014), preliminary studies with different bitter

compounds D-Salicin, Denatonium, PROP and PTC were performed. The responses of CPEC to bitter compounds were evaluated by changes in intracellular Ca^{2+} with the single-cell calcium imaging assay. As D-Salicin stimuli resulted in a variation in intracellular Ca^{2+} in CPEC (Fig. 8), we carried out experiments in the primary culture of CPEC. Salicin is an anti-inflammatory agent that is closely related in chemical make-up to aspirin and has a very similar action in the human body. When consumed by humans, salicin is metabolized into salicylic acid (Mahdi et al., 2006). Moreover, it is known that D-Salicin activates the T2R bitter receptor in humans and that Probenecid attenuates D-Salicin calcium responses by inhibition of bitter taste receptors such as Tas2R16, TAS2R38 and TAS2R43 (Meyerhof et al., 2010; Greene et al., 2011).

In our study we observed that D-Salicin showed a dose-dependent effect, with the lower calcium response corresponding to the lower concentration, 1 mM, and the major calcium response corresponding to the higher concentration, 20 mM. The use of 1 mM Probenecid, provoked a significant decrease in Ca^{2+} response in CPEC cells when stimulated with D-Salicin, in accordance with that described in the literature (Greene et al., 2011) Although our responses were not fully inhibited, a dose-related inhibition response to D-Salicin concentrations was observed. These results may indicate that Probenecid blocks specifically a subset of T2Rs but not all (Mombaerts, 2004; Greene et al., 2011; Pydi et al., 2014; Roland et al., 2014; Di Pizio and Niv, 2015; Kim et al., 2015) or that the D-Salicin response in rat is not exclusively mediated via T2R activation.

CONCLUSION

The variation in the intracellular Ca^{2+} levels after a stimuli of CPEC with D-Salicin and the expression of taste signaling molecules in the CP suggest that the bitter taste transduction pathway may be functional in CPEC cells, highlighting the capacity of the CP to perceive bitter compounds, a property mediated by bitter receptor activation.

In summary, we described the presence of the taste transduction machinery in the CP and showed that CPEC respond to a bitter compound, via bitter taste receptors. Therefore, it is likely that the taste pathway may be one of the mechanisms by which the CP surveils the chemical composition of the CSF.

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Chapter IV

Bitter taste signalling mediated by *Tas2r144* is down-regulated by 17ß-estradiol and progesterone in the rat choroid plexus

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Bitter taste signaling mediated by *Tas2r144* is down-regulated by 17β-estradiol and progesterone in the rat choroid plexus

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ABSTRACT

The blood-cerebrospinal fluid barrier is constituted by choroid plexus epithelial cells (CPEC) that regulate molecular trafficking between the blood and the cerebrospinal fluid. We hypothesize that taste receptors expressed in CPEC monitor the composition of these body fluids in a sex hormone dependent way. Thus, we compared the expression of taste related genes in the choroid plexus of sham and ovariectomized female rats, and then studied the effect of 17β-estradiol and progesterone in their expression and function. We found that the bitter receptors *Tas2r109*, *Tas2r144*, and the taste-related genes *Plcb2* and *Trpm5* were down-regulated by ovarian hormones *in vivo* and *ex vivo* with functional implications. Knocking-down *Tas2r144* with a specific siRNA in a CPEC line (Z310) effectively reduced the Ca^{2+} response to the bitter compound denatonium benzoate, in a similar manner to female sex hormones alone, suggesting that female sex hormones down-regulated the responses of CPEC to chemical stimuli by reducing *Tas2r144*.

1. Introduction

The blood-cerebrospinal fluid barrier (BCSFB) is composed by the choroid plexuses (CPs), formed by a monolayer of epithelial cells that lie over a highly vascularized stroma, floating within the brain ventricles. The CPs epithelial cells (CPEC) secrete most of the cerebrospinal fluid (CSF) (Damkier et al., 2013) and help to maintain a stable extracellular environment in the brain. Besides the barrier function itself, the CPs supply nutrients and hormones to the CSF and brain, ensure immune surveillance (Schwartz and Baruch, 2014), contribute with growth factors to the CSF that are essential for neurogenesis (Falcao et al., 2012) and more recent evidences show that the CPs also work as a peripheral clock and are a source of melatonin comparable to the pineal gland (Myung et al., 2018; Quintela et al., 2018, 2015). Chemical surveillance is another essential function of the CPs that includes the clearance and detoxification of toxic compounds (Ghersi-Egea et al., 2018b, 2018a; Johanson et al., 2011; Strazielle and Ghersi-Egea, 2016), as amyloid clearance (Pahnke et al., 2014). However, upstream regulators of this chemical surveillance system are still largely unknown. We found a large number of transcripts encoding chemosensory receptors such as olfactory and taste receptors, and demonstrated that the essential components of olfactory and taste signaling pathways are expressed and operational in the CPs (Gonçalves et al., 2016; Tomás et al., 2016). The discovery of functional taste receptors and their downstream effectors in the CP, suggests that the taste signaling pathway in the CP may be crucial for its function in the assessment of the CSF and/or blood composition, to deploy detoxification pathways and molecular trafficking within CP cells.

Taste receptors that bind sweet (Tas1R2/Tas1R3) umami (Tas1R1/Tas1R3) and bitter (Tas2rs) compounds belong to the G Protein-Coupled Receptor (GPCR) family. Upon ligand-binding they undergo a conformational change and activate a taste-specific heterotrimeric G-protein gustducin which in turn, activates phospholipase C-beta 2 (Plcb2) to produce inositol 1,4,5-trisphosphate (IP3). The IP3 opens receptor type 3 ion channels (IP3R3), provoking an increase in intracellular Ca^{2+} levels that will activate the transient receptor potential cation channel, subfamily M, member 5 (Trpm5), that eventually depolarizes the cell (Chandrashekar et al., 2006). The CPs express sweet and umami receptors, and 32 of the 36 bitter receptors (Tas2rs) expressed in rat, as well as the downstream effector proteins: α-Gust,

Abbreviations: CPs, Choroid plexus; CPEC, Choroid Plexus Epithelial Cells; CNS, Central Nervous System; CSF, Cerebrospinal Fluid; E2, 17β-Estradiol; P4, Progesterone; ER, Estrogen receptor; ERE, Estrogen Responsive Element; PR, Progesterone Receptor; BCSFB, Blood Cerebrospinal Fluid Barrier; OVX, Ovariectomized; GPCR, G Protein-Coupled Receptor; IP3, Inositol 1,4,5-trisphosphate

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Plcb2, IP3R3 and Trpm5 (Quintela et al., 2013; Tomás et al., 2016).

The CPs are multifunctional sex hormone targets (Quintela et al., 2013) containing sex hormones receptors such as progesterone receptor (PR) (Quadros et al., 2007), alpha and beta estrogen receptor (ER) (Hong-Goka and Chang, 2004) and androgen receptor (Alves et al., 2009).

Interestingly, the analysis of the effects of the sex hormone background in the rat CPs transcriptome has shown that the taste signaling pathway is one of the top five pathways regulated by female sex hormones (Quintela et al., 2013) with Tas2rs genes *Tas2r109* (taste receptor, type 2, member 109), *Tas2r124* (taste receptor, type 2, member 124), *Tas2r134* (taste receptor, type 2, member 134), *Tas2r137* (taste receptor, type 2, member 137) and *Tas2r144* (taste receptor, type 2, member 144) expressed at higher levels (at least two-fold) in the CPs of ovariectomized (OVX) female *Wistar* rats when compared to sham females (Quintela et al., 2013; Santos et al., 2017).

The taste signaling pathway was once thought to be exclusive of the oral cavity, but over the past decade, several studies clearly demonstrated different roles of this pathway. Notably, Tas2rs are expressed in several extra-oral organs, including the respiratory, urinary, digestive and immune systems, as well as in different regions of the brain, with different functional implications according to their localization (Foster et al., 2014; Lu et al., 2017; Shaik et al., 2016; Tomás et al., 2016). In barrier organs such as the airways and the gastrointestinal tract, the activation of Tas2rs triggers different types of protective responses in the organism. For instance, Tas2rs activation in the airways, evoke changes in the respiratory rate (Finger et al., 2003; Krasteva et al., 2011; Tizzano et al., 2010), regulates innate immunity (Hariri et al., 2017; Lee and Cohen, 2015a, 2015b; Lee et al., 2014; Tizzano et al., 2010), increase ciliary motility (Shah et al., 2009) and promote bronchodilation (Camoretti-Mercado et al., 2015; Deshpande et al., 2010; Lifshitz et al., 2013; Robinett et al., 2014; Tan and Sanderson, 2014). In the gastrointestinal tract, Tas2rs regulate nutrient transporter expression and nutrient uptake, the release of gut hormones and neurotransmitters involved in the regulation of energy and glucose homeostasis controlling food intake, gastric motility and ion secretion, promoting fluid secretion by the epithelium to flush the noxious irritant compounds out of the body (Avau and Depoortere, 2015; Chen et al., 2006; Jeon et al., 2008, 2011; Kaji et al., 2009; Rozengurt, 2006; Rozengurt et al., 2006).

Although Tas2rs functions have been overlooked in the brain, altered levels of Tas2rs have been found in the frontal cortex in Parkinson's disease (Garcia-Esparcia et al., 2013), in the entorhinal and frontal cortex in Alzheimer's disease, in the frontal cortex in terminal stages of Progressive Supranuclear Palsy, and in the frontal cortex and cerebellum in Creutzfeldt-Jakob disease subtypes (Ansoleaga et al., 2013). However, there are no studies defining a role for Tas2rs on the onset of central nervous system (CNS) pathologies. A number of these CNS pathologies such as Parkinson's disease (Jurado-Coronel et al., 2017), Alzheimer's disease (Li and Singh, 2014), or multiple sclerosis (Hanamsagar and Bilbo, 2016) differ between sexes in their clinical presentation, prevalence, symptoms and prognosis. Most of these pathologies present compromised CSF clearance mechanisms, which may be related to altered chemical surveillance of the CSF and/or blood (Marques et al., 2016). Understanding the regulation of taste transduction by sex hormones in the CPs may thus elucidate the mechanisms behind the chemical surveillance capacity of this organ, contributing to clarify how sex-differences may relate to CNS disease susceptibility and drug resistance.

This study focused on the regulation of key components and functioning of the bitter taste signaling pathway by female sex hormones, 17β-estradiol (E2) and progesterone (P4). We selected two taste receptors upregulated by ovariectomy, as determined by cDNA microarrays, *Tas2r109* and *Tas2r144*, and the effector proteins Plcb2 and Trpm5 (Quintela et al., 2013). The effects of E2 and P4 on the regulation of these genes in rat CPs, and the molecular mechanisms behind their regulation were analyzed in terms of gene expression (RT-qPCR) and functionality (calcium imaging) after bitter taste signaling activation.

2. Experimental procedures

2.1. Animals

All the experiments on rats were handled in compliance with the NIH guidelines, and the European Union rules for the care and handling of laboratory animals (Directive, 2010/63/EU). Adult female Wistar rats (8–10 week old) were used to monitor the effect of female sex hormones on the expression of *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* genes. All the animals were housed in appropriate cages at constant room temperature (Sealsafe Individual Ventilated Cages Blue Line – 1291H, coupled to an Air Handling Unit Touch Slim Plus, 20 ± 2 °C, 55 ± 10% humidity, Tecniplas, Italy) in a 12 h light-12 h dark period and given standard laboratory chow (Diete Standard, 4RF21, Mucedola, Italy) and water *ad libitum*. To investigate the mechanisms involved in the E2 or P4 regulation on taste signaling pathway genes, CPs were dissected from newborn Wistar rats (P6–P7) and treated with E2 and P4 as described below. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Research Centre of the University of Beira Interior.

2.2. In vivo studies: ovariectomy and CPs collection

Proestrous adult female *Wistar* rats at the age of 8 10-weeks were either sham-operated or OVX and, two weeks after surgery animals were euthanized under anesthesia with a ketamine/medetomidine mixture. Both lateral ventricular CPs were collected and frozen in liquid nitrogen for total RNA extraction (n = 5 independent experiments for each experimental group).

2.3. Ex vivo studies: rat CPs explants

CPs were collected from newborn *Wistar rats* directly to 48 well plates containing Dulbecco's Modified Eagle Medium-high glucose without phenol red (DMEM, Sigma-Aldrich) supplemented with 10% charcoal stripped FBS (Sigma-Aldrich) and 100 U/mL of penicillin/streptomycin (Sigma-Aldrich), with three different concentrations of E2 (Sigma Aldrich; 1, 10 and 100 nM) or P4 (Calbiochem; 1, 10 and 100 nM) and incubated for 6 h in a humidified incubator in 95% air with 5% CO_2 at 37 °C.

To assay if regulation of taste signaling genes were mediated by ERs or PR, CPs were incubated in E2 (1 nM) or P4 (1 nM) for 6 h, in the presence or absence of fulvestrant (ICI; Sigma Aldrich; 100 nM), or mifepristone (RU486; Sigma Aldrich; 10 μM), the antagonists of ERs and PR, respectively. Antagonists were added 90 min before E2 or P4 incubation. Control conditions were set with vehicle (0.002% ethanol) only, and with ICI and RU486 alone. After that, CPs were collected directly to TRI reagent (Sigma-Aldrich) for total RNA extraction. For each condition, a pool of two CP from different animals was used, n = 5 independent experiments for each experimental group.

2.4. mRNA expression analysis in rat CPs

Total RNA was extracted from CPs using TRI reagent (Sigma Aldrich) according to the manufacturer's instructions. After quantitation of RNA with a NanoPhotometer (Implen, Munich, Germany), its integrity was assessed by 1% agarose gel electrophoresis. Then, 1 μg of RNA was DNAse I (Sigma Aldrich) treated and reverse transcribed using M-MuLV Reverse Transcriptase (RT) (NZYTech, Ltd) according to the manufacturer instructions. Analysis of the mRNA expression levels of *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* and of the reference gene *Cyclophilin A* was carried out by real time RT-qPCR using the KAPA

Table 1
Sequences of the primers and cycling conditions used in real-time RT-qPCR.

RefSeq	Gene name	Gene symbol	Primer sequence 5′-3′	Exon (bp)	Size (bp)	AT (°C)
NM_001080939.1	*Taste receptor, type 2, member 109*	***Tas2r109***	Fw: CTGGTGTCGCTGGTCTTACT Rv: GCAGTCTGCACGGTAATGGT	NA (424 443) NA (530 549)	126	60
NM_001025150.1	*Taste receptor, type 2, member 144*	***Tas2r144***	Fw: CGCGTGGCTCAATGTCTTCT Rv: GAGCCGAGGCATCAACACTAC	NA (333 352) NA (412 432)	100	60
NM_053478.1	*Phospholipase C, beta 2*	***Plcb2***	Fw: AGCAGTTCAACCCCTTCTCG Rv: GCAGTAGGTGACAGCTTGGT	19 (2106 2125) 20 (2267 2286)	181	60
NM_001191896.1	*Transient receptor potential cation channel, subfamily M, member 5*	***Trpm5***	Fw: ATTTTCCGCCGTGTGCTCTA Rv: CAACGGGTGAAGAGAGCAGT	19 (2704 2723) 20 (2786 2805)	102	60
NM_017101.1	*Cyclophilin A*	***Ppia***	Fw: CAAGACTGAGTGGCTGGATGG Rv: GCCCGCAAGTCAAAGAAATTAGAG	4 (339 413) 5 (532 555)	119	60

AT-Annealing Temperature; Fw-Forward; Rv-Reverse; bp-base pair; NA-Not Applicable.

SYBR® FAST qPCR Kit (Kapa Biosystems) and the CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Cycling parameters were the following: 95 °C for 10 min and 40 cycles at 95 °C for 30 s, 30 s at the annealing temperature for the respective gene and 72 °C for 30 s. The relative expression of the studied genes was determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Pfaffl, 2001). Each sample was analyzed in triplicate. For *in vivo* experiments data from each animal was normalized to sham levels and an average of 5 animals was presented. The sequence of the primers, fragment length and the respective cycling conditions for the genes analyzed are presented in Table 1.

2.5. In vitro studies: Z310 cell culture and siRNA knockdown

The rat CPs epithelial cell line Z310 was a generous gift by Dr. Wei Zheng (Purdue University, West Lafayette, USA). Z310 cells were cultured in DMEM-high glucose without phenol red with 10% FBS (Biochrom AG, Berlin) and 100U/mL of penicillin/streptomycin (Sigma Aldrich) (Zheng and Zhao, 2002). The culture medium was replaced every two days until 90% confluence was achieved followed by serum deprivation for 24 h. To analyze the effect of E2 and P4 in the response of Z310 cells to a bitter stimulus, different hormone concentrations (1, 10 and 100 nM of E2 or P4) in DMEM-high glucose supplemented with 10% charcoal stripped FBS and 100 U/mL of penicillin/streptomycin (Sigma Aldrich) were used to incubate the cells, with and without the respective antagonists (ICI or RU486), as described above. Then, the Z310 cell line responses to a bitter stimulus were analyzed by intracellular calcium imaging experiments.

For *Tas2r144* knockdown, cells were seeded in 8 well Lab-Tek (Thermo Fisher Scientific) at a density of 1×10^4 cell/well in DMEM-high glucose containing 10% charcoal stripped FBS and 100 U/mL of penicillin/streptomycin. Then, after 48 h, the cells were transfected with 10 nM of a mixture of small interfering RNA (siRNA) targeting the *Tas2r144* (s178904, Silencer® Select, Ambion) and 5 μL of Lipofectamine® 2000 Transfection Reagent (Invitrogen) in Opti-MEM medium (Invitrogen), following the manufacturer's instructions. As a control for *Tas2r144* specific targeting, a Silencer® Select negative control siRNA (4390843, Ambion) was also used at 10 nM. After 24 h, the hormones E2 (1 nM) or P4 (1 nM) were added and the cells were further incubated for 24 h before single cell calcium imaging using the bitter stimuli.

2.6. Single-cell calcium imaging

Single cell calcium mobilization assays were carried out as described earlier (Tomás et al., 2016). Z310 cells were seeded at 1×10^4 cells/well in 8-well Lab-Tek (Thermo Fisher Scientific) chambers and placed in a humidified incubator in 95% air 5% CO_2 at 37 °C. Before stimulation with the bitter compounds, Denatonium benzoate (DB) (Sigma Aldrich) and Epigallocatechin gallate (Acros Organics), cells were treated as follows: cells were loaded for 1 h at 16 °C with 5 μM Fura-2 AM (Molecular Probes, USA) 0.1% BSA and 0.02% pluronic acid F-127, in culture medium. After a wash step with Tyrode's solution (NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 2 mM, Na-pyruvate 10 mM, glucose 10 mM, HEPES 10 mM, $NaHCO_3$ 5 mM, pH 7.4), cells were incubated for 30 min in this solution. The 8-well chambers were placed on an inverted fluorescence microscope (Axio Imager A1, Carl Zeiss). Before each stimulus, a 2 min baseline was acquired and then cells were stimulated with 1, 2.5, 5 and 10 mM of DB or 0.01, 0.015, 0.025, 0.05 and 0.1 mM of Epigallocatechin gallate for 15 min. The involvement of bitter taste receptors (Tas2rs) in the responses obtained to DB was evaluated using a stimuli of 5 and 10 mM DB in the presence of the Tas2rs blocker, Probenecid (1 mM, Sigma Aldrich) in Tyrode's solution, as well as by the silencing of the *Tas2r144*.

To study the effect of E2 and P4, Z310 cells were pre-incubated for 24 h in DMEM-high glucose containing 10% charcoal stripped FBS and 100 U/mL of penicillin/streptomycin, with different hormone concentrations (E2: 1, 10 and 100 nM; P4: 1, 10 and 100 nM; E2 + P4: 1 nM of each) and stimulated with 5 mM of the bitter compound DB, as described above. To determine if cell responses depend on ERs or/and PR, similar Ca^{2+} imaging assays were done after the pretreatment with E2 1 nM or P4 1 nM in the presence of ICI 100 nM and RU486 10 μM, respectively. The same experiments were also performed with ICI or RU486 alone. Receptor antagonists were added 90 min before the hormone incubation. Control conditions (nontreated cells) were set up with vehicle (0.002% ethanol) only.

The intracellular concentration-dependent Ca^{2+} changes were evaluated by quantifying the ratio of the fluorescence emitted at 520 nm following alternate excitation at 340 nm and 380 nm, using a Lambda DG4 apparatus (Sutter Instruments, Novato, CA) and a 520 nm bandpass filter (Carl Zeiss) under a 40× objective (Carl Zeiss) with an AxioVision camera and software (Carl Zeiss). Data was processed using the Fiji software (MediaWiki). Results are presented as an average of the changes in intracellular Ca^{2+} levels of 15–20 cells from three or more independent experiments.

2.7. MTT assay

Z310 cell viability upon hormonal treatments was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to discard any toxic effects of the hormones. The MTT assay is a standard, widely used method of measuring cell viability (Mosmann, 1983). Briefly, Z310 cells were seeded at a density of 5×10^3 cells per well, in a 96-well plate, and 24 h after seeding the medium was replaced by serum-free culture medium for 24 h. Cells were then incubated during 24 h in DMEM-high glucose without phenol red containing 10% charcoal stripped FBS and 100 U/mL of penicillin/streptomycin with the respective hormone concentration (E2: 1, 10 and 100 nM; E2 1 nM + ICI 100 nM; P4: 1, 10 and 100 nM; P4 1 nM + RU486 10 μM; ICI 100 nM; RU486 10 μM; vehicle alone: ethanol 0.002%). Then, 20 μL of MTT solution 5 mg/mL in PBS was added to each well and plates were incubated for 4 h at 37 °C in a

humidified atmosphere containing 5% CO_2. Untreated cells and ethanol 70% treated cells were used as negative and positive controls to assess cytotoxicity, respectively. Following incubation, the culture medium was removed and the formazan crystals were dissolved in 150 μL of DMSO. The absorbance was read at 570 nm.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Statistical significance was considered for p values < 0.05 using Students T-test, One-Way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test and Two-Way ANOVA followed by Sidak's multiple comparisons test.

3. Results

3.1. Ovariectomy up-regulates the expression of taste-related genes

The effects of female sex hormones on the expression of the taste-related genes were analyzed by real time RT-qPCR in the CPs of OVX and sham female rats. There was a clear increase of the expression of *Tas2r109* (2.5 fold), *Tas2r144* (2.2 fold), *Plcb2* (2.2 fold) and *Trpm5* (1.3 fold) genes (Fig. 1) in the CPs of OVX rats two weeks after ovariectomy, compared to sham-operated animals.

These expression differences have a similar magnitude to those obtained by analysis of our cDNA microarrays data (Quintela et al., 2013) (mRNA expression ratio of OVX versus Sham) (Table 2).

3.2. E2 and P4 down-regulate the expression of taste-related genes in CP explants

Consistent with the *in vivo* data, dissected CPs from newborn rats incubated with increasing concentrations of E2 (1, 10 and 100 nM), P4 (1, 10 and 100 nM) or E2 + P4 (1 nM of each), for 6 h, showed reduced expression of *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* genes, for all the hormone concentrations analyzed (Figs. 2–4). The involvement of ER or PR in the expression of taste-related genes was analyzed in CPs explants treated with E2 or P4, in the presence or absence of the ER and PR antagonists ICI or RU486, respectively. E2 treatment (1 nM) led to relevant decreases in gene expression: *Tas2r109* (2.3 fold), *Tas2r144* (3.2 fold), *Plcb2* (2.1 fold) and *Trpm5* (1.6 fold) (Fig. 2). The *Tas2r109* and *Plcb2* attained its minimum at 100 nM E2 (decreases of 4.8 and 3.8 fold, respectively), the *Tas2r144* at 1 nM (3.2 fold) and the *Trpm5* gene was equally reduced for all the E2 concentrations studied (≈1.5 fold). The E2-induced decrease in *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* mRNA levels were attenuated in the presence of ICI (Fig. 2).

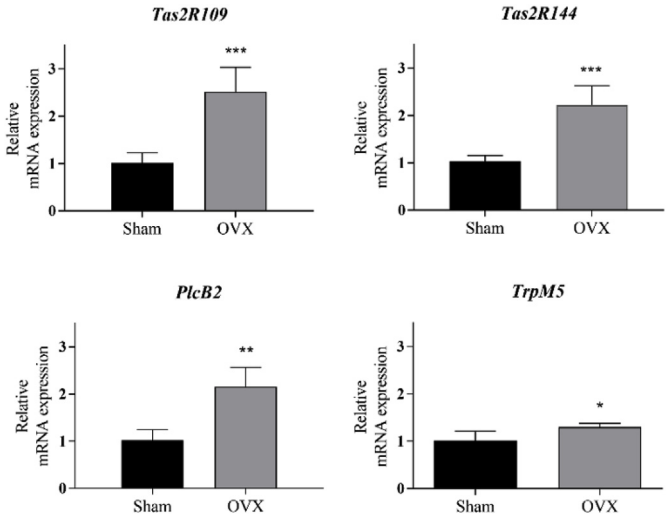

Fig. 1. Effect of ovariectomy on *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* gene expression in female rat's CPs. The mRNA levels were compared between sham and ovariectomized (OVX) female rats by real time RT-qPCR. Results are presented as mean ± SEM, n = 5 independent experiments. Students t-test, *p < 0.05; **p < 0.01; ***p < 0.001.

Table 2
Fold changes of taste signaling pathway genes obtained by real time RT-qPCR and by cDNA microarray (sham vs OVX).

Gene		*Tas2r109*	*Tas2r144*	*Plcb2*	*Trpm5*
Methodology	RT-qPCR	2.5***	2.2***	2.2**	1.3*
	Microarrays (Quintela et al., 2013)	1.5**	3.3**	1.9**	1.7**

T-test *p < 0.05, **p < 0.01, ***p < 0.001.

With the 1 nM P4 treatment, decreases in the expression of *Tas2r109*, *Plcb2* and *Trpm5* in the same order of magnitude were observed (Fig. 3; ≈1.8 fold) and similar down-regulation effects were observed for all P4 concentrations tested (1, 10 and 100 nM). *Tas2r144* was differentially affected by P4 concentration attaining its minimum at 1 and 10 nM with decreases in expression of 4.5 and 4.3 fold, while P4 100 nM decreased its expression only 1.6 fold. However, the receptor antagonist RU486 did not prevent the P4 effects (Fig. 3), in spite of that for the *Tas2r144* gene the presence of the PR blocker resulted in a significant increase of the gene expression, but this was still significantly lower than in the control.

The combined effect of both hormones was also evaluated in an assay with E2 and P4, showing reduction in the expression of *Tas2r109* (3.6 fold), *Tas2r144* (4.5 fold), *Plcb2* (1.9 fold) and *Trpm5* (1.7 fold) genes (Fig. 4), but an effective synergistic down-regulation was only observed for the *Tas2r109* gene when compared with the P4 treatment alone (1.8 fold).

3.3. Denatonium benzoate elicits Ca^{2+} responses in the CPs cell line Z310 via Tas2r144

Functional assessment of the E2 and P4 effects on the activation of bitter taste receptors was carried out using the Z310 cell line that is a well characterized model for CPEC studies. Beforehand, the analysis of the E2 (1, 10, 100 nM), P4 (1, 10, 100 nM), ICI (100 mM), RU (10 μM), E2 (1 nM) + ICI (100 mM), P4 (1 nM) + RU (10 μM) and vehicle effects in the viability of Z310 cells was assessed by the MTT assay. The results obtained (Supplement, Fig. A1) showed that none of the conditions used affected cell viability.

Previous work carried out in our laboratory had shown that primary cultures of CPEC responded to the bitter compound D-Salicin (Tomás et al., 2016). However, there was no evidence that the CP cell line Z310 responded to any bitter stimuli. Thus, we stimulated these cells with two different bitter compounds, DB and Epigallocatechin gallate, both known *Tas2r144* ligands (Lossow et al., 2016), and measured intracellular Ca^{2+} variations. Single cell calcium imaging was performed in Z310 cells with increasing concentrations of DB (1, 2.5, 5 and 10 mM) and Epigallocatechin gallate (0.01, 0.015, 0.025, 0.05 and 0.01 mM) (Fig. 5). However, while increasing concentrations of DB caused increasing Ca^{2+} responses (Fig. 5A), the same was not observed for the Epigallocatechin gallate stimuli (Fig. 5B). In addition, the Ca^{2+} variation in Z310 cells was higher in response to DB than to Epigallocatechin gallate stimuli. Therefore, we choose to use the DB stimuli in the following studies. To investigate if this response occurred via Tas2rs, we first used Probenecid, a Tas2rs blocker (Greene et al., 2011), which promoted a significant reduction in Ca^{2+} responses ($\Delta F = 0.82 \pm 0.02$ and $\Delta F = 1.10 \pm 0.13$) after stimulation with 5 mM and 10 mM of DB respectively, when compared to Ca^{2+} responses without Probenecid ($\Delta F = 1.53 \pm 0.31$ and $\Delta F = 2.82 \pm 0.31$, respectively) (Fig. 5). To analyze if *Tas2r144* mediates the response of

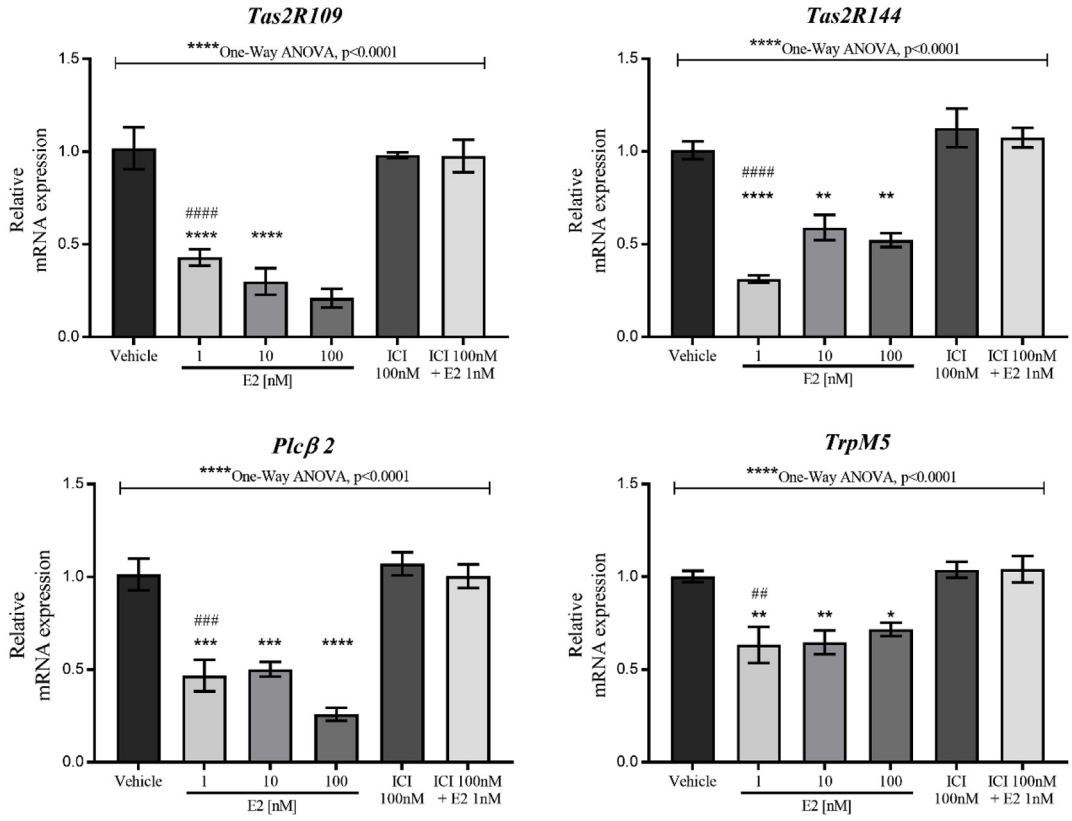

Fig. 2. Effect of increasing concentrations of E2 on the expression of taste-related genes in rat CPs' explants in the presence or absence of the receptor antagonist ICI 100 nM. One-Way ANOVA, followed by Tukey's multiple comparisons test against control, n = 5 independent experiments, *relatively to vehicle, #relatively to ICI + E2 1 nM (*p < 0.05, **/##p < 0.01, ***/###p < 0.001, ****/####p < 0.0001; E2 17β-estradiol; ICI fulvestrant).

Z310 cells to DB, a specific siRNA was used to knockdown the *Tas2r144*. A 74% decrease of Ca^{2+} responses to DB occurred in knocked-down cells when compared to control conditions (vehicle only) (Fig. 6).

3.4. Ca^{2+} responses of the CP cell line Z310 to DB are reduced by E2 and P4

Having established that the taste-related genes are down regulated by E2 and P4 in CPs' explants, we proceeded with functional studies in different hormonal backgrounds. For that, Z310 cells, pre-treated with increasing concentrations of the hormone (E2 and/or P4), were stimulated with the bitter taste compound DB and the intracellular Ca^{2+} responses were measured. A DB (5 mM) stimulus in Z310 cells pre-treated with 1 nM E2 showed a reduction in intracellular Ca^{2+} release of about 50%, when compared to non-treated cells (50.1 ± 11.6% vs 100.0 ± 8.8%, respectively; Fig. 7A). Concurrently, a similar DB stimulus in Z310 cells pre-treated with P4 also presented a significant decrease of about 40–50% in Ca^{2+} release, compared to non-treated cells (50.7 ± 7.2%, 61.2 ± 10.9% and 60.5 ± 20.5%, for 1; 10 and 100 nM of P4, respectively vs 100.0 ± 8.8%; Fig. 8A). None of these hormonal downregulation effects were dose-dependent. In an attempt to clarify the molecular mechanisms behind that regulation, the putative role of the steroid receptors ER or PR was explored. Similar Ca^{2+} imaging experiments were performed in the presence of the antagonists of ER (ICI) and or PR (RU486). The decreased Ca^{2+} response to DB after treatment with E2 (1 nM) was abolished by ICI pre-treatment in Z310 cells, while ICI treatment alone had no significant effects on Ca^{2+} response, compared to untreated cells (Fig. 7A). On the other hand, the decrease in the response to DB elicited by P4 treatment was not attenuated by RU486. In fact, the treatment with RU486 + P4 1 nM decreased the Ca^{2+} response by ≈ 42.5% which was similar to the one obtained with 1 nM P4 (≈51%). The treatment with RU486 alone did not alter the Ca^{2+} response to DB (Fig. 8A).

The hormonal treatment with E2 and P4 down-regulates expression of the *Tas2r144* gene (Figs. 3 and 4) and the response of Z310 cells to DB is mediated by *Tas2r144* (Fig. 6). Taking that into account, Z310 cells knocked down for *Tas2r144* were incubated with E2 and P4 and stimulated with DB to evaluate if these hormones could further reduce the Ca^{2+} variation when *Tas2r144* was silenced. The results obtained (Figs. 7B and 8B) did not show any additional effect of the hormones in these conditions.

4. Discussion

In a recent study, we reported the expression of genes of the canonical taste signaling pathway in CPs, and demonstrated its functionality by observing responses in primary cultures of CPEC to the bitter compound D-Salicin (Tomás et al., 2016). In addition, data from a CPs microarray study showed that the decline of hormone levels in female rats upon OVX clearly induced up-regulation of genes of this pathway (Quintela et al., 2013), including the bitter taste receptors *Tas2r109, Tas2r124, Tas2r134, and Tas2r144,* and the downstream effector molecules *Plcb2* and *Trpm5*. Moreover, two bitter receptors differentially expressed between female and male *Wistar* rats (*Tas2r109* and *Tas2r144*) have higher expression in males (GSE87045).

In this study, we hypothesized that Tas2rs activation is one of the mechanisms involved in the chemical surveillance capacity of the CPs, which might be of relevance for monitoring the chemical composition of blood and CSF and that this mechanism is regulated by the female sex hormones, E2 and P4.

In support of this hypothesis, we demonstrated that ovariectomy up-regulates expression of *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5*, in the CPs of female rats, suggesting that female sex hormones down-regulate their expression. Then, we demonstrated through *ex-vivo* studies with CPs

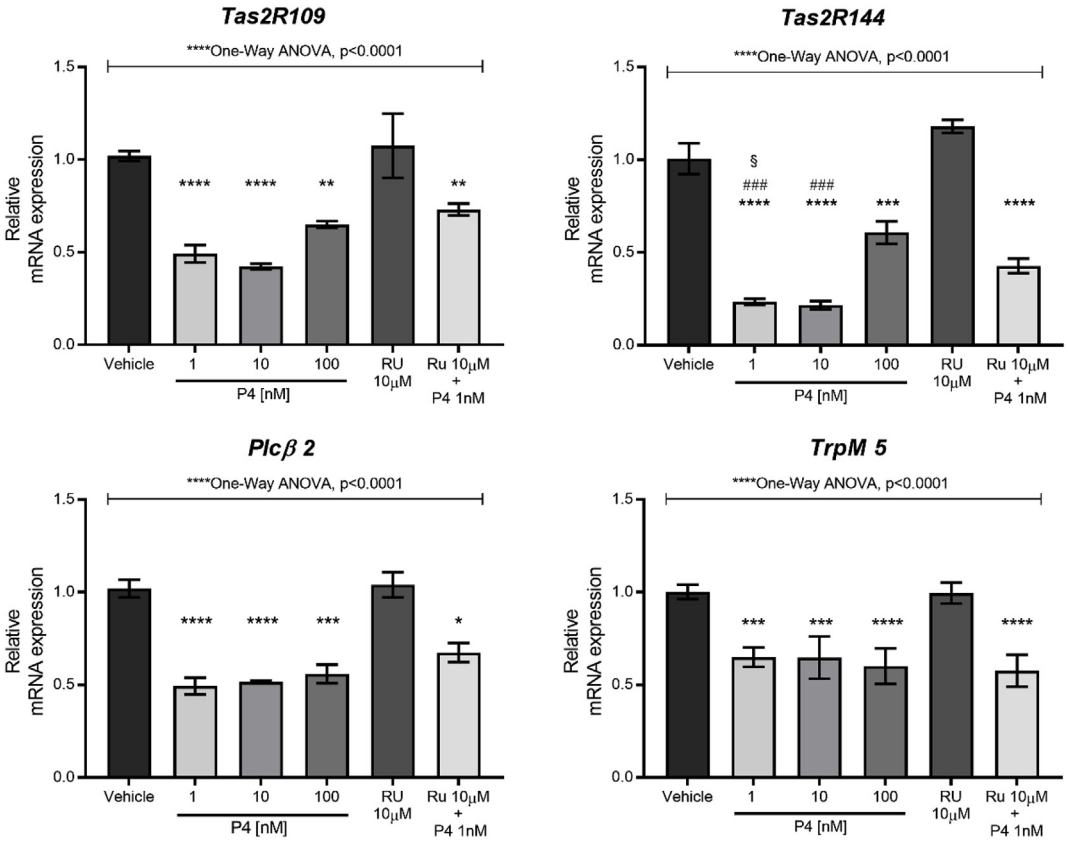

Fig. 3. Effects of increasing concentrations of P4 on the expression of taste-related genes in rat CPs' explants in the absence or in the presence of the receptor antagonist RU486 10 μM. One-Way ANOVA, followed by Tukey's multiple comparisons test against control, n = 5 independent experiments, *relatively to vehicle, #relatively to P4 100 nM, §relatively to RU + P4 1 nM (*/§p < 0.05, **p < 0.01, ***/###p < 0.001, ****p < 0.0001; P4 – progesterone; RU – mifepristone).

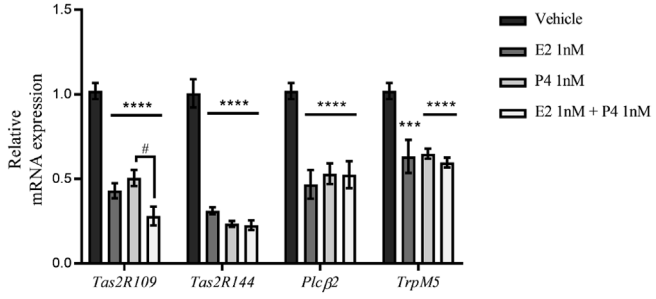

Fig. 4. Effects of E2 1 nM + P4 1 nM on taste-related gene expression in rat CPs explants. Two-Way ANOVA, followed by Sidak's multiple comparisons test, n = 5 independent experiments, *relatively to vehicle, #P4 Vs E2 + P4 (#p < 0.05, ***p < 0.0001, ****p < 0.001 (E2 – 17β-estradiol; P4 – progesterone).

explants, that in fact these genes are down-regulated by E2 and P4 and further explored the involvement of their cognate receptors in their regulation. Our data demonstrated that the down-regulation exerted by E2 on the expression of these genes is mediated by ERs, as pre-treatment of CPs with the ERs antagonist, ICI, abrogated the effect of E2 on *Tas2r109*, *Tas2r144, Plcb2* and *Trpm5* genes. However, the down regulation exerted by P4 was not mediated by the classical nuclear PR, as the effect observed was not reverted in the presence of the classical nuclear PR antagonist, RU486. Also, our results suggest that the differential expression observed by Quintela et al. (2013) in basal expression of *Tas2r109* and *Tas2r144* between CPs of female and male rats is due to female sex hormones, as E2 and P4 diminished *Tas2r109* and *Tas2r144* expression *ex vivo*.

Functional implications of E2 and P4 in the detection of a bitter compound by CPs was assessed *in vitro,* in Z310 cells, using DB and Epigallocatechin gallate in Ca^{2+} imaging assays. Both compounds

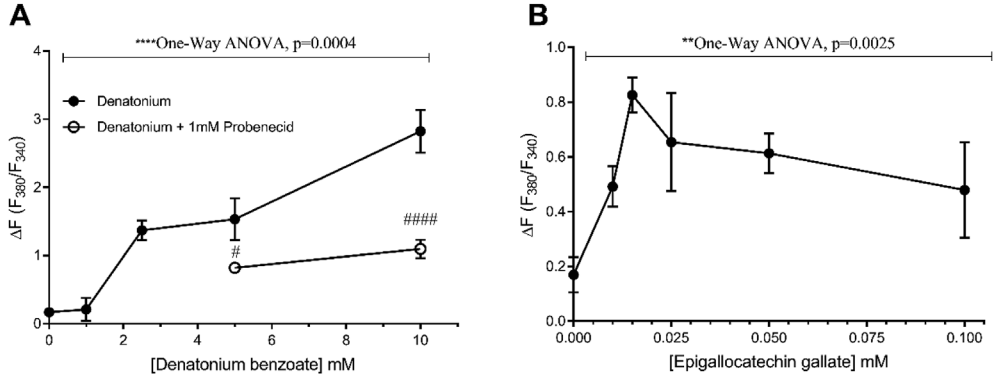

Fig. 5. Bitter stimuli elicits calcium responses in the CPs' Z310 cell line. A) Ca^{2+} responses of the rat Z310 cells to DB (1, 2.5, 5 and 10 mM), in the absence (●) and in the presence (○) of Probenecid (1 mM). B) Ca^{2+} responses of the rat Z310 cells to Epigallocatechin gallate (0.01, 0.015, 0.025, 0.05 and 0.01 mM). Results are presented as mean ± SD, n = 3 independent experiments. One-Way ANOVA, ****p < 0.0001, **p = 0.0025; Two-Way ANOVA followed by Sidak's multiple comparisons test #p = 0.0105, ####p < 0.0001.

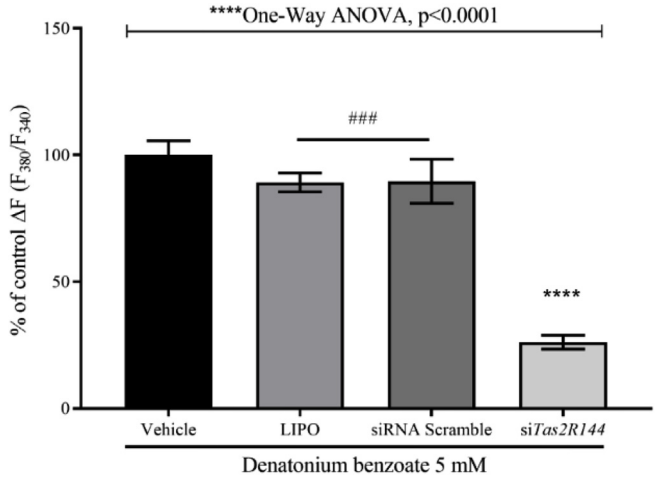

Fig. 6. Intracellular Ca^{2+} responses of the rat Z310 cell line transfected with siRNA targeting the *Tas2r144* stimulated with 5 mM DB. One-Way ANOVA, followed by Tukey's multiple comparisons test against control (vehicle), n = 4 independent experiments, *relatively to vehicle, # relatively to si*Tas2r144* (****p < 0.0001, ###p = 0.0001; LIPO- Lipofectamine).

elicited a significant intracellular Ca^{2+} response by cells, however while the response to DB was dose dependent, the response to Epigallocatechin gallate was not. The Ca^{2+} response was significantly diminished in the presence of the Tas2rs blocker Probenecid for DB 5 mM and 10 mM of about 46% and 60%, respectively, suggesting that the CP cell line's response to DB was mediated by Tas2rs. Previously, we found that Probenecid can also inhibit the response to D-salicin in primary cultures of CP cells (Tomás et al., 2016). Probenecid is known to inhibit the responses of human TAS2R16, TAS2R38 and TAS2R43 to D-salicin, 6-propyl-3-thiouracil (PROP), N-phenylthiourea (PTC) and aloin (Greene et al., 2011). Other researchers have found that Probenecid can also inhibit the mouse sperm's response to PTC (Xu et al., 2013). However, to be certain that DB triggers a response in Z310 cells via bitter taste receptors, we selected *Tas2r144* that binds DB (Lossow et al., 2016) to knock-down with a specific siRNA. Z310 cells with silenced *Tas2r144* showed a decrease of 74% in the Ca^{2+} response after DB stimuli, concluding that the response of Z310 cells to DB is mediated by the bitter receptor *Tas2r144*. Consistent with our *in vivo* and *ex vivo* results, incubation of Z310 cells with E2 and P4 or both, affected the Ca^{2+} response of Z310 cells diminishing it by approximately 40%. The results obtained by silencing *Tas2r144* together with the hormonal treatment suggest that the effect of sex hormones depends on the regulation of the receptor itself. As no enhancement of the reduction in Ca2+ was observed with the combination of receptor silencing and hormonal treatment. However, as in *ex vivo* experiments, the Ca^{2+} response of Z310 cells to DB was abolished by the ER antagonist, ICI, but not by the PR antagonist RU486, confirming the involvement of the ER but not the classical nuclear PR.

It is known that the interaction of E2 with ERs may lead to classical mechanisms: upon binding to the receptor, activated ER homodimerizes or heterodimerizes, and bind to the Estrogen Responsive Element (ERE) of specific genes, regulating the expression of target genes (Hall et al., 2001). ICI is an ER antagonist which blocks and degrades ERs, thereby reducing cellular levels of ER protein and promoting the complete blockage of ER-regulated genes (Dowsett et al., 2005; Wakeling, 2000). Nuclear ERs (ERα and ERβ) are present in several tissues including the CPs (Hong-Goka and Chang, 2004; Kuiper et al., 1997) where they act as transcriptional factors to modulate the expression pattern of target genes. Indeed, the *Tas2r109* and *Trpm5* genes contain an ERE in their proximal promoters at position 742 (tttgGTCAggttatgttta) and 146 (aaggGTCAggttggtgcca), respectively (www.genomatix.de). Thus, our data suggest that the down-regulation of the *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5* genes by E2 in CPs is partially mediated by ER activation in the nucleus. However, the effect of E2 observed in *Tas2r144* and *Plcb2* expression and its annulment in the presence of ICI, despite the absence of EREs (www.genomatix.de) may indicate a possible involvement of the membrane bound ER (mER). The mER is present in the CP (Santos et al., 2017), can also be blocked by ICI and its activation may have impact in gene expression (Kelly and Rønnekleiv, 2009; Song et al., 2005). The down-regulation of the studied taste-related genes by P4 was not abolished by pre-treatment of CPs with the classical nuclear PR antagonist, RU486, suggesting that the results observed are not mediated by the classical nuclear P4 receptor. However, they may result from the binding of P4 to its membrane receptor (mPR). mPR is also expressed in the CP (Pang et al., 2013). Still, which PR receptor contributes to the observed effect in taste-related genes needs to be further explored in the future. Nonetheless, the observed results indicate that the expression of taste-related genes is up-regulated with the decline of ovarian hormones.

Our CP findings are consistent with other studies showing that alterations in female sex hormones modulate the taste function in the oral cavity. Clear differences in taste perception processing between males and females, in humans and rodents are evident, indicating that the same gustatory stimuli produce differential input to the brains of males and females (Di Lorenzo and Monroe, 1990, 1989; Martin and Sollars, 2017). Even with several contradictory data, it is generally accepted that oral perception of flavors, in the taste buds, changes during pregnancy (Choo and Dando, 2017; Deglaire et al., 2015; Faas et al., 2010). Consistent results show that the bitter taste sensitivity increases during pregnancy in humans (Duffy et al., 1998; Ochsenbein-Kolble et al., 2005) suggesting a role of female sex hormones in its regulation. Moreover, the response of the parabrachial pons to the bitter compound quinine is increased in OVX rats when compared to controls (Di Lorenzo and Monroe, 1990). Thus, it seems likely that female sex hormones affect the capacity to respond to bitter compounds both at the level of

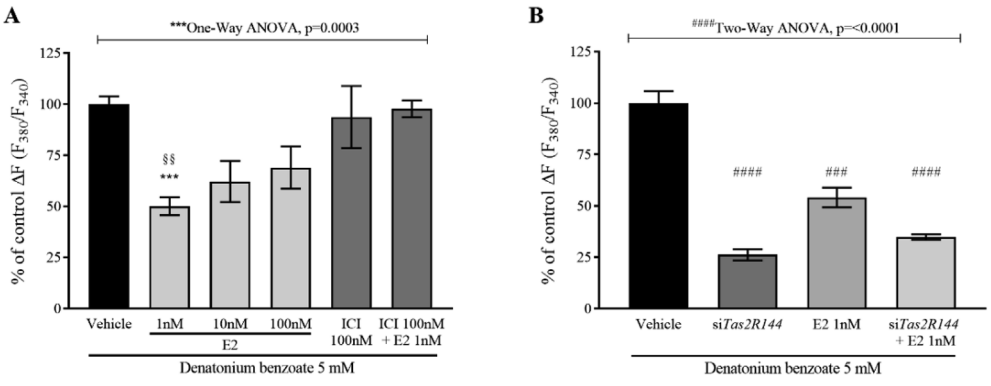

Fig. 7. Intracellular Ca^{2+} imaging responses of Z310 cells to Denatonium benzoate (5 mM) in different hormonal backgrounds. A) Z310 cells intracellular Ca^{2+} variation after treatment with E2 (1, 10 and 100 nM) with and without pre-treatment with the ER antagonist ICI 100 nM. B) Z310 cells intracellular Ca^{2+} variation after *Tas2r144* silencing and treatment with E2 1 nM. Results are presented as mean ± SEM, n = 5 independent experiments. One-Way ANOVA, followed by Tukey's multiple comparisons test, *relatively to control, §relatively to ICI 100 nM + E2 1 nm (***p < 0.0003, §§p < 0.005). Two-Way ANOVA, followed by Sidak's multiple comparisons test, # relatively to control (####p < 0.0001, ###p = 0.0004; E2 17β-estradiol; ER estrogen receptor; ICI fulvestrant).

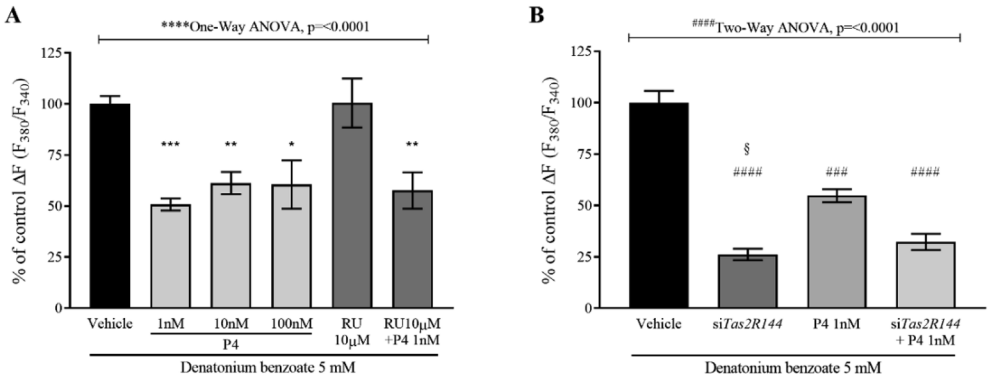

Fig. 8. Intracellular Ca^{2+} imaging responses of Z310 cells to DB (5 mM) in different hormonal backgrounds. A) Z310 cells intracellular Ca^{2+} variation after treatment with P4 (1, 10 and 100 nM) with and without pre-treatment with the PR antagonist RU486 10 μM. B) Z310 cells intracellular Ca^{2+} variation after *Tas2r144* silencing and treatment with P4 1 nM. Results are presented as mean ± SEM, n = 5 independent experiments. One-Way ANOVA, followed by Tukey's multiple comparisons test, *relatively to control (***p = 0.0003, **p = 0.008, *p = 0.0151). Two-Way ANOVA, followed by Sidak's multiple comparisons test, #relatively to control, §relatively to P4 ($^{\#\#\#\#}p < 0.0001$, $^{\#\#\#}p = 0.0001$, $^{\S}p = 0.0207$; P4 – progesterone; PR – progesterone receptor; RU – mifepristone).

taste buds as well as in the CP, in a similar way.

In extra-oral tissues, the taste transduction pathway is described as a monitoring system of the surrounding fluids that continues to sense nutrients and noxious substances implicated in diverse biological processes (Dalesio et al., 2018). Moreover, Tas2rs are responsible for the recognition of a large number of structurally distinct toxic compounds (Chandrashekar et al., 2006) and because common drugs are bitter compounds and thus effective ligands for Tas2rs, extra-oral Tas2rs have been proposed as mediators of off-target drug effects (Clark et al., 2012). For example, the bitter compounds of beer seem to play a role in preventing dementia in an Alzheimer's model mice by suppressing neuroinflammation and improving cognitive function (Ano et al., 2017) and the activation of Tas2rs, in the gut, increases the efflux activity of the ATP Binding Cassette (ABC) transporter ABCB1 (Jeon et al., 2011). ABC transporters in CPs are responsible for the efflux of toxic compounds from brain or CSF into blood, thus contributing to the blood–CSF–barrier function and to the clearance of potential toxic blood and brain borne compounds (Bernstein et al., 2014; Strazielle and Ghersi-Egea, 2016). Strikingly, TAS2Rs are differential expressed in post-mortem brains from patients with Alzheimer's, Parkinson's and Creutzfeldt-Jakob diseases (Ansoleaga et al., 2013; Garcia-Esparcia et al., 2013), which also have different clinical presentations between sexes (Jurado-Coronel et al., 2017; Li and Singh, 2014). Thus, Tas2rs in the CPs may be targets for therapeutic drugs for brain diseases with compromised CSF clearance. However, the role of Tas2rs in CPs remains poorly understood and needs to be further studied, but it is now possible to assume that Tas2R activation in the CPs have functional implications and that they are regulated by female sex hormones.

5. Conclusion

In conclusion, our findings indicate a functional regulation of the bitter taste signaling in CPs by female sex hormones. In particular, we show that E2 and P4 down-regulate the expression of *Tas2r109, Tas2r144, Plcb2* and *Trpm5* genes. These E2 and P4 effects seem to be mediated by ERs (and/or mER) and by the mPR respectively. We also report that the functional bitter taste receptor, *Tas*2r144, that responds to DB stimuli in CP cells, is inhibited by female sex hormones. The taste chemosensory signaling may be an essential component of the CPs chemical surveillance apparatus, to detect alterations in CSF or blood composition, and elicit responses to modulate and maintain brain homeostasis. All these studied regulation mechanisms may contribute to better understand the differences seen in the onset and progression of CNS diseases between sexes that may be also related to CPs functional differences between males and females.

Conflicts of interest

The authors declare no competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2019.110521.

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Appendix 1

Fig. A.1

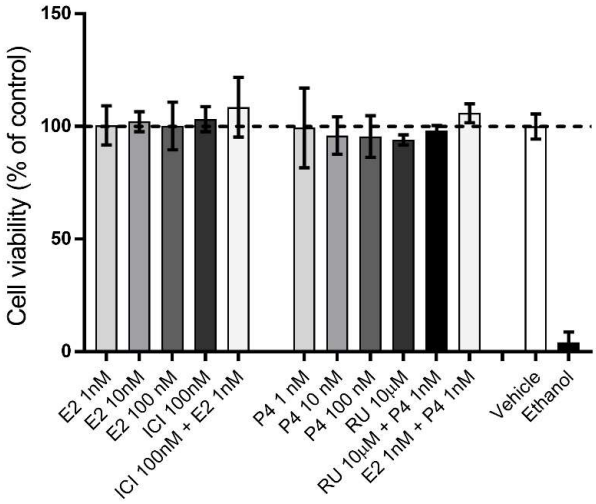

Fig. A.1 - MTT assays conducted in Z310 CPs cell line after a 24h incubation with increasing concentrations (1, 10 and 100 nM) of E2 and P4. Untreated cells and ethanol 70% treated cells were used as negative and positive control to cytotoxicity, respectively. Mean percentage values are relative to the untreated cells (100%), 3 independent experiments are shown. ANOVA, mean ±SD. CPs - choroid plexus; E2 - 17ß-estradiol; P4 - progesterone; ICI - fulvestrant; RU - mifepristone.

Fig. A.2

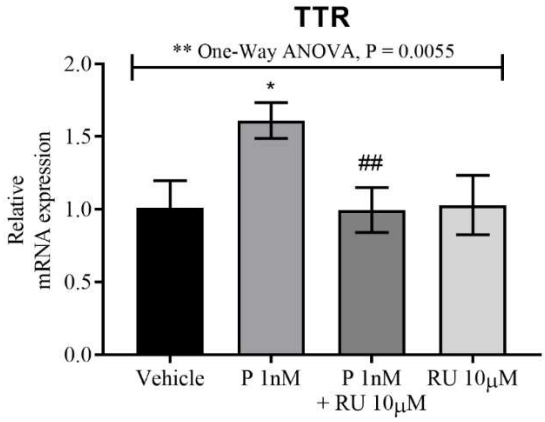

Fig. A.2 - Effects of 10nM P4 on the expression of TTR gene in rat CPs' explants in the absence or in the presence of the receptor antagonist RU486 10µM. One-Way ANOVA, followed by Tukey´s multiple comparisons test against control, n = 3 independent experiments, *relatively to vehicle, #relatively to P4 100 nM (*p=0.011, ##p=0.0095; P4 - progesterone; RU - mifepristone).

Chapter V

General Conclusions and Further Perspectives

General Conclusions and Further Perspectives

The CPs located in brain ventricles are constituted by a complex network of capillaries lined by a single layer of epithelial cells resting on a basal membrane. At the apical membrane CP cells contact the CSF and in the basal side the blood. The CPs have various crucial functions in maintaining the normal CNS homeostasis and in the assessment of the chemical composition of blood and CSF. However, the mechanisms behind the monitoring ability of CPs remain to be elucidate. The finding of chemical detection pathways in CPs, such as the taste signalling pathway, raised the hypothesis that this pathway could be one of the mechanisms behind the monitoring capacity of CP at the BCSF. A proper chemical detection mechanism in CPs is crucial to protect the CNS against harmful microorganisms and toxins present in blood and/or CSF.

Our study started by evaluating if all the molecules involved in the taste signalling cascade, as the taste GPCRs and the downstream signalling molecules, were present in rat CPs and in primary cultures of CPECs from newborn (3-5 days old) *Wistar* rats (Chapter 3). The results showed that transcripts for the taste GPRCs *Tas1r1*, *Tas1r2*, *Tas1r3*, *Tas2r109*, *Tas2r144* and for the downstream signalling molecules *Gnat3*, *Gnb3*, *Plcb2*, *Ip3r3*, *Trpm5* and *Scnng1* were present in primary cultures of CPECs and in rat CPs. Furthermore, we assessed the expression of proteins from different steps of the taste signalling cascade such as the taste GPCRs T1R2/T1R3 (sweet receptor), T1R1/T1R3 (umami receptor) and Tas2r144 – involved in detection of compounds; Gnat3 and Plcb2 – mediators of the signal transduction; and Trpm5 that will depolarize the cell and promote signal propagation. The results obtained corroborate the results reported by Ren and co-workers which reported the expression of *Tas1r2* and *Tas1r3* in CPs for the first time (Ren et al., 2009) and the results obtained by a cDNA microarray study previously carried out by our work group that identified taste-related genes in the CPs of rats (Quintela et al., 2013). In addition, besides proving that the genes encoding the taste pathway are expressed in the CP, we have shown for the first time that the taste machinery is present at the plasma membrane of CPEPC, where it can work as a potential chemical detection system.

The evaluation of taste signalling pathway functionality in CPs was split between the two experimental works presented and as mentioned before, we focussed our attention in the bitter taste pathway. The activation of the bitter pathway was carried out in primary cultures of CPEC using the bitter compound D-Salicin (CHAPTER 3), and in the immortalized cell line of CPEC, the Z310 cell line, with Denatonium Benzoate and Epigallocatechin Gallate as stimuli (CHAPTER 4). Both stimuli evoked a cellular response, which was diminished in the presence of a T2Rs blocker and by silencing specific receptors. Our results point out that the taste signalling pathway makes use of T2R receptors in the chemical surveillance of bitter compounds in the CSF.

Finally, in order to achieve the last objective of the present work, the regulation of taste-related genes in rat CPs by sex hormones was examined (CHAPTER 4). Our results uncovered the regulation

of taste-related genes by female sex hormones. Specifically, E2 and P4 down-regulated the taste-related genes *Tas2r109*, *Tas2r144*, *Plcb2* and *Trpm5*. However, while E2 effects seem to be mediated by ERs (and/or mER), P4 effects seem to be mediated only by mPR.

Functional studies showed a decreased response of CP cells to the bitter stimulus Denatonium Benzoate in the presence of E2 and/or P4, probably as a result of the decreased expression of taste-related proteins. In particular, the response of CP cells to the bitter stimulus, Denatonium Benzoate, was proven to be mediated via the bitter receptor Tas2r144. The decreased response of CP cells to Denatonium Benzoate via Tas2r144 in the presence of sex hormones is an indication that these hormones may alter the CP detection of the surrounding chemical composition by regulating the bitter signalling pathway.

Nonetheless, the location of T2Rs still needs to be further evaluated, to determine which receptors are apically or basal located in order to better understand which T2Rs are involved in the entrance or clearance of chemicals from the brain.

In conclusion, our studies demonstrated the presence of the taste signalling machinery and a functional bitter signalling pathway regulated by sex hormones in the CPs. The results obtained along this study give support to our initial hypothesis that taste signalling may be an essential component of the CPs chemical surveillance to detect alteration in CSF or blood composition and elicit responses to modulate and maintain brain homeostasis.

The presence of a functional bitter signalling in CPs cells, supports the existence of an upstream mechanism to deploy appropriate downstream responses upon ligand-binding affecting cell differentiation, proliferation, migration, chemiotaxis, endocrine responses, cytoskeleton remodelling, as seen in other non-gustatory organs. Thus, it is of greatest importance to further explore the function of these receptors at the BCSFB as they represent a promising route for manipulating the entrance or clearance of many chemicals in the brain. For instance, it is known that T2R activation results in the up-regulation of the multidrug resistance protein ABCB1 compromising the action of chemotherapeutics agents in pancreas cancer (Gaida et al., 2016) and limiting the absorption of toxic substances in the gut (Jeon et al., 2011). The study of the involvement of T2Rs in the regulatory mechanism of multidrug efflux transporters may contribute to better understand the regulatory mechanism behind chemoresistance. Because, the major problem observed for several agents in the treatment of brain disorders is chemoresistance associated to the overexpression of multidrug efflux transporters in the blood brain interfaces (Feldmann and Koepp, 2016), the study of the effect of T2Rs in multidrug efflux transporters at the CP, may contribute to better clarify the regulatory mechanism behind chemoresistance. T2Rs also bind several other therapeutic agents rather than chemotherapeutics agents, such as chloroquine (antimalarial), dextromethorphan (antitussive) or haloperidol (antipsycothic) (Clark et al., 2012). Nevertheless, one of the biggest problems in CNS pathologies is the presence of therapeutic agent in therapeutic concentrations inside the CNS because most therapeutic agents are blocked by brain barriers (BSCFB and BBB) and reach the CNS in very low concentrations unable to perform their therapeutic function. The activation of T2Rs by therapeutic agents can be a target

of study in order to increase their bioavailability in the CNS. Moreover, the finding that female SH regulates T2Rs opens a window to more personalised therapies and may contribute to better understand the differences seen in the onset and progression of CNS diseases between genders.

Although, the results obtained point to specific responses to different stimuli, the response of CP cells to bitter stimuli still need to be further explored. It is generally accepted that a bitter stimulus can elicit bitter signalling activation throughout one or more T2R having each receptor different threshold concentrations and responses profile considering the stimuli tested and the T2R activated (Lossow et al., 2016). The crosstalk's between different taste receptors and between olfactory and vomeronasal receptors (Santos et al., 2019), also present in CPs, should also be addressed in future studies.

5.1. References

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